



Genetic Approaches to Study Human Embryonic Stem Cell Self-Renewal and Survival

Citation

Tajonar, Adriana. 2012. Genetic Approaches to Study Human Embryonic Stem Cell Self-Renewal and Survival. Doctoral dissertation, Harvard University.

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:9453702>

Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

© 2011 – Adriana Tajonar

All rights reserved.

GENETIC APPROACHES TO STUDY HUMAN EMBRYONIC STEM CELL SELF-RENEWAL AND SURVIVAL

Abstract

Embryonic stem (ES) cells can be maintained indefinitely in culture while retaining the ability to give rise to cellular derivatives from the three germ layers. These unique characteristics hold great promise for regenerative medicine and underscore the importance of understanding the molecular mechanisms behind ES cell maintenance. The embryonic stem cell state is supported by a delicate equilibrium of mechanisms that maintain pluripotency, prevent differentiation, and promote proliferation and survival. We sought to find genes that could contribute to one or more of these processes in human ES cells by using a gain-of-function screen of over 8000 human open reading frames (ORFs). We identify Vestigial-like 4 (Vgll4), a co-transcriptional regulator with no previously known function in ES cells, as a positive regulator for survival of human ES cells. Specifically, Vgll4 protects human ES cells from dissociation stress, and enhances colony formation from single cells. These effects may be attributable in part to the ability of Vgll4 to decrease the activity of initiator and effector caspases. Based on global transcriptional analysis, we hypothesize that Vgll4 enhances survival of hES cells at clonal densities by regulating changes in the cytoskeleton, which may in turn regulate pathways known to result in hES cell death.

This dissertation introduces a novel approach for studying hES cell survival in the context of cell dissociation and presents Vgll4 as a novel regulator of this process. We also propose that Vgll4 could have multiple functions in hES cells including possible roles in pluripotency, cell cycle dynamics, Hippo pathway regulation, and TGF β signaling. A direct regulator of survival in human embryonic stem cells could have important implications for facilitating the generation of transgenic cell lines and reporters, thus harnessing the therapeutic application of these cells.

Table of contents

Abstract	iii
Table of contents	v
List of figures.....	ix
List of tables	xv
Acknowledgements.....	xvi
Chapter 1 Introduction	1
1.1 <i>History of pluripotent cells.....</i>	2
1.1.1. Teratocarcinoma and embryonal carcinoma cells	2
1.1.2. Mouse and human embryonic stem cell derivation	3
1.2 <i>Mouse embryonic stem cell self-renewal</i>	5
1.3 <i>Human embryonic stem cell self-renewal and survival</i>	7
1.3.1. Self-renewal mechanisms in hES cells.....	7
1.3.2. Survival mechanisms in hES cells	10
1.4 <i>Induced pluripotent stem cells</i>	12
1.5 <i>Genetic screening approaches to understanding self-renewal.....</i>	14
1.6 <i>Some current challenges and questions in the field.....</i>	16
1.7 <i>Overview of the dissertation.....</i>	18
References.....	19
Chapter 2 Genetic gain-of-function screen for self-renewal genes	25
2.1 <i>Introduction</i>	25
2.2 <i>Primary screen.....</i>	29
2.2.1. Experimental Design	29
2.2.2. Results.....	35
2.3 <i>Secondary screen</i>	43
2.3.1. Introduction.....	43
2.3.2. Experimental Design	43
2.3.3. Results.....	49
2.4 <i>Discussion.....</i>	55
2.5 <i>Materials and Methods used in this chapter</i>	58

2.5.1.	Culture of hES cells	58
2.5.2.	Lentivirus production	58
2.5.3.	hES cell transduction.....	59
2.5.4.	Flow cytometry analysis	60
2.5.5.	Immunofluorescent staining.....	60
2.6	<i>Note regarding author contributions</i>	61
	<i>References.....</i>	61

Chapter 3 Vgll4 is a novel regulator of survival in human embryonic stem cells.....65

3.1	<i>Introduction</i>	65
3.1.1.	Vestigial-like proteins are transcriptional co-regulators that mediate the activity of Transcriptional Enhancer Factors	65
3.1.2.	The Rho/Rock pathway prevents the death of hES cells upon dissociation	66
3.2	<i>Characterization of Vgll4 expression</i>	68
3.2.1.	Expression of Vgll4 in hES cells.....	68
3.2.2.	Effects of Vgll4 overexpression at the global transcriptional level	70
3.3	<i>Vgll4 overexpression results in faster doubling time of pluripotent cell populations</i>	75
3.4	<i>Vgll4 protects pluripotent cells from dissociation stress.....</i>	77
3.4.1.	Vgll4 maintains a higher proportion of live cells	77
3.4.2.	Vgll4 suppresses Caspase activity.....	81
3.4.3.	Vgll4 is necessary to regulate survival and caspase activity of hES cells	83
3.5	<i>Vgll4 confers an advantage in colony forming potential from single cells.....</i>	87
3.6	<i>A possible interaction between Vgll4 and the Rho/Rock pathway for promoting hES cell survival</i>	90
3.7	<i>Conclusions and Discussion.....</i>	92
3.8	<i>Methods used in this chapter.....</i>	96
3.8.1.	Culture of hES cells	96
3.8.2.	Embryoid body differentiation.....	96
3.8.3.	Real-time PCR analysis	96
3.8.4.	Microarray analysis	97
3.8.5.	Growth curve analysis	97
3.8.6.	Apoptosis analysis by Annexin V and PI.....	98
3.8.7.	shRNA knockdown.....	98
3.8.8.	hES cell transduction.....	99
3.8.9.	Clonality assay	99

3.8.10.	Immunofluorescent staining	100
3.8.11.	Vgll4 custom-made antibody	101
3.8.12.	Luminescence assays for caspase activity	101
3.9	<i>Note regarding author contributions</i>	102
	<i>References</i>	102

Chapter 4 Elucidating the role of Vgll4 in human embryonic stem cells 104

4.1	<i>Introduction</i>	104
4.2	<i>A role for Vgll4 in the pluripotency network</i>	105
4.2.1.	Rationale	105
4.2.2.	Results	105
4.2.3.	Conclusions	121
4.3	<i>A role for Vgll4 in the regulation of proliferation</i>	124
4.3.1.	Rationale	124
4.3.2.	Results	124
4.3.3.	Conclusions	127
4.4	<i>A role for Vgll4 in the Hippo pathway</i>	129
4.4.1.	Rationale	129
4.4.2.	Results	131
4.4.3.	Conclusions	135
4.5	<i>A role for Vgll4 in the regulation of TGFβ signaling</i>	137
4.5.1.	Rationale	137
4.5.2.	Results	137
4.5.3.	Conclusions	140
4.6	<i>Concluding remarks</i>	140
4.7	<i>Methods used in this chapter</i>	142
4.7.1.	hES cell culture	142
4.7.2.	Assessment of pluripotency markers by flow cytometry	142
4.7.3.	Real-time PCR throughout EB differentiation	143
4.7.4.	Endoderm and cardiac mesoderm differentiation	144
4.7.5.	Teratoma formation	144
4.7.6.	shRNA knockdown	145
4.7.7.	Reprogramming into iPS cells	145
4.7.8.	Cell cycle dynamics by flow cytometry	146
4.7.9.	Mitotic cell assessment by immunofluorescence	146
4.7.10.	Hippo pathway activation	147
4.7.11.	Microarray analysis	148
4.7.12.	Assessment of TGF β signaling	148
4.8	<i>Note regarding author contributions</i>	149
	<i>References</i>	149

Chapter 5 Concluding Discussion and Future Directions..... 153

<i>References</i>	160
-------------------------	-----

List of figures

Figure 1-1. Self-renewal mechanisms differ between mouse and human ES cells.	
Mouse ES cells can be maintained by LIF (acting through STAT3) and BMP (inducing Id genes) in the primed state. In the naïve state (indicated in blue), inhibition of ERK and GSK-3 β allow the maintenance of mES cells in the ground state. Human ES cells are maintained by Activin/TGF β (which act through Smad2/3 and promote Nanog expression) and FGF. FGF and Activin promote each other's expression. Additionally, FGF might participate in the inhibition of BMP through an unknown mechanism. BMP promotes differentiation in hES cells by inhibiting Nanog expression. Oct4, Sox2, and Nanog orchestrate a pluripotency transcriptional program in both species.	9
Figure 1-2. Cell dissociation promotes apoptosis in hES cells.	
E-cadherin-mediated cell-to-cell junctions inhibit Abr. When cell-to-cell junctions are disrupted, Abr activates Rho and inhibits Rac. Rho activates Rock, which phosphorylates myosin, leading to myosin hyperactivation and blebbing). Myosin hyperactivation sets off caspase-mediated apoptosis. (Figure adapted from [41]).....	11
Figure 2-1. A gain-of-function strategy to discover novel genes involved in the maintenance of hES cells.	
HUES6 cells transduced with lentiviruses carrying a hORFeome library containing approximately 8000 genes (Table S1) were subjected to three weeks of treatment with 10 μ M TGF β inhibitor (TGF β i). Colonies with characteristic ES cell morphology after such treatment were picked and their DNA was sequenced to identify 75 preliminary candidates	28
Figure 2-2. A Gateway-adapted pHAGE vector to efficiently promote gene expression in hES cells.	
The hORFeome library is contained in a lentiviral vector. The gene of interest is driven by an EF1 α promoter and the ORF is fused to a hemmagglutinin (HA) tag. In addition, this lentiviral vector carries a puromycin resistance gene driven by a PGK promoter. Primer binding sites are indicated by black block arrows under the construct.	28
Figure 2-3. Inhibition of TGFβ signaling results in loss of hES cell colony morphology and pluripotency marker expression.	
Cells transduced with a Nanog ORF were used as a positive control, cells transduced with a GFP ORF and wildtype cells were used as negative controls. a) Negative controls lost the characteristic hESC morphology after three weeks or 3 passages of SB-431542 (TGF β i) treatment (Bars = 500 μ m). b) Cells transduced with GFP and treated with TGF β i for three weeks downregulate Tra1-60 and SSEA-3 (not shown). However, cells transduced with Nanog under this treatment retain expression of these markers.....	30
Figure 2-4. EF1α and PGK promoters drive efficient expression in hESCs.	
HUES6 cells were transduced with EF1 α -GFP. Before puromycin selection cells that were not transduced are visible. After selecting with 2 μ g/ml of puromycin for 2 days, a homogenous population of GFP-expressing cells is observed, indicating efficient activity of both promoters in hES cells.	34
Figure 2-5. Optimization of conditions for secondary verification assay.	
A TGF β inhibitor (SB-431542), an FGF inhibitor (SU-5402), and Retinoic Acid were	

tested for their ability to cause differentiation in 5 or 7 days. Differentiation was assessed by evaluating colony morphology, Oct4, and Tra 1-60 expression. At both timepoints, FGF β i and Retinoic Acid had a mild effect. Treatment with TGF β i caused some differentiation, but a considerable number of undifferentiated cells were still present at both timepoints. The combination of TGF β i and FGF β i caused robust loss of pluripotency marker expression and colony morphology by 5 days.....	45
Figure 2-6. Verification of secondary screening conditions using overexpression controls. Combined inhibition of TGF β and FGF causes Oct4 downregulation and loss of colony morphology (as shown by DAPI panel) in cells overexpressing GFP but not in cells overexpressing Nanog. GFP control cells show little differentiation after 5 days of treatment with TGF β i or FGF β i alone.	47
Figure 2-7. Identification of four positive hits using a secondary verification assay. Preliminary candidates were verified using a more stringent test for maintenance of the hES cell state. The verification assay consisted of 5 days of treatment with 10 μ M TGF β i in addition to 20 μ M FGF inhibitor (FGF β i). DMSO was used as a vehicle control. After this treatment, qualitative analysis of colony morphology and expression of ES cell markers Oct4 and Tra 1-60 was assessed by immunostaining. Hits indicated with an asterisk here, and in Table 1-1, were those that were identified after the secondary verification assay.....	48
Figure 2-8. Individual verification of preliminary candidates reveals positive hits for pluripotency maintenance. Three independent rounds of candidate verification are shown. Images show Oct4 staining in the samples treated with the combination of inhibitors for five days. We also included other pluripotency-related genes that were not originally in our list of preliminary candidates. We performed a qualitative evaluation of colony morphology and proportion of Oct4 expression to identify positive hits. Vgll4 consistently retains Oct4 expression and colony morphology. An asterisk marks the genes identified as hits. Chmp4a was part of verification round 4.....	50
Figure 2-9. Vgll4 is a co-transcriptional regulator that can maintain hES cell identity after treatment with inhibitors. Cells transduced with Vgll4 or control genes were treated with vehicle control or with the inhibitor mix for 5 days and stained for Oct4 at the end of the treatment. Quantification of Oct4 $^{+}$ cells after treatment using Cellomics image acquisition and software (DMSO: vehicle control).....	52
Figure 2-10. Vgll4 maintains expression of pluripotency markers after treatment with TGFβi and FGFβi. Cells overexpressing Vgll4 and treated with the combination of inhibitors for 5 days are able to maintain expression of a panel of pluripotency markers including transcription factors Oct4, Sox2, and Nanog as well as surface markers like Tra 1-60.....	53
Figure 3-1. Vgll4 is expressed in pluripotent and differentiated cells. a) Expression of Vgll4 relative to GAPDH in pluripotent cells (WT HUES6) and across an embryoid body (EB) differentiation timecourse. The days of differentiation are indicated after “d” in the X axis. b) Immunostaining of Oct4 and Vgll4 in hES cells. Vgll4 is detected in the Oct4-positive and some Oct4-negative cells. c) Expression of Nanog relative to GAPDH in pluripotent cells and EBs. d) Expression of Oct4 relative to GAPDH in pluripotent cells and EBs.....	69
Figure 3-2. Vgll4 causes downregulation of genes involved in apoptosis and in Rho-Rock pathway activation. a) Graphical representation of microarray data	

showing the relative expression changes for pro-apoptotic genes in Vgll4 relative to WT hES cells. The q-values for these changes in expression are: Caspase 9 q = 7.29%; TNFRSF25 q = 7.67 %; TNFRSF10B q = 2.44%; APAF1 q = 3.35%; BCL2L1 = 4.61%. b) Graphical representation of microarray data showing the relative expression changes for genes involved in Rho-Rock signaling in Vgll4 relative to WT hES cells. The q-values for these changes in expression are: RHOB q = 2.34%, ARGEF3 q = 2.75%, ARRB1 q = 7.67%. Q-values were obtained by analysis with SAM.....	74
Figure 3-3. Pluripotent cells overexpressing Vgll4 have a higher population-doubling rate in maintenance conditions. Graphs depicting the growth rate (k) using the exponential growth formula $N = N_0 e^{kt}$ where N= final number of cells, N_0 = initial number of cells, t = time after plating in days. The data was plotted on a log-normal scale and a linear fit was performed. a) Human embryonic stem cell lines overexpressing Nanog, Tubulin, or Vgll4 and maintained in self-renewing conditions. Eight timepoints were analyzed for HUES6, seven for HUES1, and three for HUES8. b) Human induced pluripotent stem cell lines overexpressing Nanog, Tubulin, or Vgll4 and maintained in self-renewing conditions. Seven timepoints were analyzed for iPS RBd and six for iPS 18a.	76
Figure 3-4. Annexin V and PI staining can be used to detect live, apoptotic, and dead cell populations in hES cells. a) Vgll4- and Tubulin-transduced hES cells either untreated or treated with dissociation buffer for 12-18 hours and stained with Annexin V-APC and PI, then analyzed by FACS. Live cells are APC ⁻ , PI ⁻ ; apoptotic cells are APC ⁺ , PI ⁻ , and dead cells are PI ⁺ . b) Etoposide (ETO)-treated single-color controls for APC and PI are used to accurately set gates for the experiment.....	79
Figure 3-5. Vgll4 promotes survival and inhibits apoptosis in response to cell dissociation. hES cells overexpressing Vgll4 or Tubulin were treated with a Hank's-based enzyme-free dissociation buffer or 500 μ M EDTA or 10 μ M etoposide (ETO) for 12-18 hours. The proportion of live, apoptotic, and dead cells was assessed by Annexin V and PI staining. Results represent flow cytometry data from 6 independent experiments. Error bars represent the standard deviation and the p-values were obtained with an unpaired Student's T-test.	80
Figure 3-6. Vgll4 decreases Caspase 3/7 and Caspase 9 activity. a) Caspase 3/7 and b) Caspase 9 activity for Vgll4- and Tubulin-hES cells after treatment with dissociation conditions. Both were assessed using caspase luminescence assays. Relative luminescence units (RLU) represent the signal after subtracting the background. Background was obtained by mixing medium with assay buffer. Error bars represent the standard deviation of triplicates. P-values were obtained using an unpaired Student's T-test.....	82
Figure 3-7. V78 is a shRNA that achieves 60% knockdown of Vgll4. Vgll4 expression levels relative to GAPDH in cells transduced with a shRNA against Vgll4 (V78) or a scrambled control (mir neg). V78-1 A and B represent different virus production batches and transductions.....	83
Figure 3-8. Vgll4 knockdown increases hES cell sensitivity to dissociation conditions. hES cells stably expressing a scrambled control (mir neg), V78, or Vgll4 were treated with a Hank's-based enzyme-free dissociation buffer, 500 μ M EDTA or 10 μ M etoposide (ETO) for 12-18 hours. The proportion of live,	

apoptotic, and dead cells was assessed by Annexin V and PI staining. Results represent flow cytometry data from 3 independent experiments. Error bars represent the standard deviation and the p-values were obtained with an unpaired Student's T-test.....	85
Figure 3-9. Vgll4 knockdown increases caspase activity upon treatment with dissociation conditions. a) Caspase 3/7 and b) Caspase 9 activity for mir neg, V78, and Vgll4-hES cells after treatment with dissociation conditions. Both were assessed using Caspase-Glo luminescence assays. Relative luminescence units (RLU) represent the signal after subtracting the background. Error bars represent the standard deviation from triplicate samples. P-values were calculated using an unpaired Student's T-test.....	86
Figure 3-10. Vgll4 increases the colony-forming efficiency of hES cells. a) hESCs transduced with Nanog, GFP or Vgll4 were plated at single cell densities on 96-well plates and counted 10 days after plating. Percentage of wells where colonies formed is shown for conditions with and without Rock inhibitor. b) Results for low-density plating at 1000, 100, 10 and 1 cells per well. Errors bars represent the standard error from 2 experiments.....	88
Figure 3-11. Clonally-derived cells maintain expression of pluripotency markers and lentiviral genes. After 10 days in culture, colonies derived from single cells were fixed and analyzed for the expression of the pluripotency marker Oct4 and the lentivirus-encoded GFP (a) or Vgll4-HA (b).	89
Figure 3-12. A possible interaction between Vgll4 and the Rho/Rock pathway promotes survival of hES cells. Colony formation efficiency for 100 cells/well treated with the indicated concentrations of Rock inhibitor. a) Rock inhibitor dosage response curve in control cells (mir neg). b) Colony formation efficiency of control (mir neg) and Vgll4-knock-down (V78) cells. V78 are deficient in their colony-forming ability. c) Full Vgll4 functionality is required to benefit from Rock-inhibition. V78 colony-forming efficiency is shown normalized to the colony-forming efficiency of mir neg. Increasing concentrations of Rock inhibitor are unable to rescue for Vgll4-deficiency at non-saturating concentrations. d) Model for mode of action of Vgll4. Rho signaling is activated upon disruption of E-cadherin cell-to-cell contacts resulting in apoptosis. Vgll4 could inhibit cell death by modulating the expression of apoptosis and cytoskeleton genes. Alternatively, VGLL4 could have a role in the cytoplasm independent from its role as a co-transcriptional regulator in the nucleus.....	91
Figure 4-1. Vgll4 delays the loss of pluripotency markers in response to long-term treatment with SB-431542. Human ES cells were treated with SB-431542 (TGFβi) at 10μM for the number of passages indicated and assayed for the expression of pluripotency markers by flow cytometry. a) Expression of Tra1-60 over time. b) Expression of SSEA-3 over time. These results represent one experiment.....	108
Figure 4-2. Vgll4 delays loss of colony morphology upon inhibition of TGFβ and FGF signaling. Cells were treated with 10 μM SB-431542 and 20 μM SU-5402 (TF) for 7 days. Changes in colony morphology were monitored for Vgll4-hES cells and WT hES cells by light microscopy.	109
Figure 4-3. Vgll4 delays the loss of pluripotency markers during embryoid body differentiation. Embryoid bodies were formed from WT hES cells or hES cells transduced with Nanog, Vgll4, or GFP. mRNA levels of a) Oct4 and b) Nanog were assessed by real time PCR throughout a timecourse of 15 days. Levels of	

expression are relative to a GAPDH control. The blue bar represents endogenous expression of the gene in wildtype hES cells. P-values were calculated from Ct values using an unpaired Student's T-test.....	111
Figure 4-4. Overexpression of Nanog or Vgll4 is not sufficient to prevent the loss of Oct4 expression under directed differentiation protocols. Human ES cells overexpressing Nanog, Vgll4 or GFP were subjected to differentiation into a) endoderm or b) cardiac mesoderm progenitors. Oct4 expression was assessed at the end of the differentiation protocols.	112
Figure 4-5. Human ES cells overexpressing Vgll4, Nanog, or GFP give rise to endoderm progenitors at similar efficiencies. Human ES cell lines were treated to induce differentiation into endoderm progenitors. The efficiency of differentiation was assessed by staining for Sox17 and FoxA2.....	113
Figure 4-6. Nanog, Vgll4 or GFP overexpression are permissive to differentiation into the three germ layers by teratoma formation. Human ES cells overexpressing Nanog, Vgll4, or GFP were injected subcutaneously into immunocompromised mice. Derivatives from the three germ layers were identified by hematoxylin/eosin staining. Gl: glandular epithelium (endoderm), Gt: gut-like epithelium (endoderm), Ne: neural epithelium (ectoderm), M: melanocytes (ectoderm), C: cartilage (mesoderm).	115
Figure 4-7. Expression levels of pluripotency factors respond to Vgll4 downregulation. The levels of Vgll4 were reduced by approximately 60% using V78, a shRNA against Vgll4. Nanog and Oct4 expression were assessed by real-time PCR. a) Nanog mRNA levels in cells treated with a scrambled control (mir neg) or V78. b) Oct4 mRNA levels in cells treated with a scrambled control (mir neg) or V78. A and B represent two independent viral production rounds and transductions. P-values were calculated from Ct values using an unpaired Student's T-test.....	116
Figure 4-8. Downregulation of Nanog levels lead to an increase in Vgll4 expression. a) shRNAs (N52, N53, N55) against Nanog achieve 30-80% reduction in Nanog mRNA levels. b) Levels of Vgll4 were assessed in response to Nanog knockdown with the indicated shRNAs.....	117
Figure 4-9. Distribution of cell populations in the cell cycle. Two representative experiments (a and b) are shown. Distribution of cells in the cell cycle was assessed by DNA quantification using PI staining. The Dean-Jett-Fox model was used for calculating the area under the curve. The reason behind G1, S, G2 percentages adding up to more than 100% is that the modeling algorithms contain some approximations and are rounded numbers.	126
Figure 4-10. Proportion of mitotic cells in self-renewing hES cell lines. Human ES cell lines were fixed 5-6 days after plating and stained with Oct4, Human Nuclear Antigen (HuNu), phospho-histone H3 (pH3) and DAPI. Panels to the left show representative images of mitotic cells and quantification is provided to the right. Significance was calculated using an unpaired Student's T-test. N.S. = not significant.	126
Figure 4-11. Vgll4 inhibits TEAD-mediated transcription in a reporter assay. 293T cells carried the reporter plasmid outlined in a). This reporter contains TEAD binding sequences in front of a luciferase gene. b) Cells were transfected the reporter plasmid and Renilla as a transfection control in addition to the indicated genes. Activation of the reporter was assessed as a function of luciferase/Renilla luminescence. Mock = treated only with transfection	

reagents, luc/R = transfected only with reporter plasmid and Renilla control, S127A = constitutively active Yap. Results represent 3 biological replicates in duplicates. P-value was obtained with an unpaired Student's T-test.	133
Figure 4-12. Vgll4 downregulates Hippo pathway target genes in hES cells. Relative expression changes for pro-apoptotic genes in Vgll4 relative to WT hES cells were obtained by microarray analysis. The q-values for these changes in expression are: CTGF q = 0%; CYR61 q = 0%. Q-values were obtained by analysis with SAM.....	134
Figure 4-13. Vgll4 inhibits the activation of a gene target of TGFβ signaling. a) TMLC reporter cells carry a portion of the plasminogen activator inhibitor 1 (PAI-1) driving expression of a luciferase gene. b) TMLC lines overexpressing GFP or Vgll4 were treated with the indicated treatments for 12 hours and luciferase activity was assessed.....	139

List of tables

Table 2-1 - Candidates from primary ORFeome screen.....	36
Table 3-1. Cellular processes with a significant response to Vgl14 overexpression in hES cells.....	72
Table 3-2. Functional networks with a significant response to Vgl14 overexpression in hES cells.....	73

Acknowledgements

This dissertation would not have been possible without the people below. My heartfelt thanks to:

Doug Melton, for being my advisor all these years and giving me your trust. For inspiring me to think with no limits and allowing me to pursue interesting scientific questions. Thank you for standing by me in crucial times during my grad school experience.

Dena Cohen for your advice, support, and for believing in me. For always keeping your door open and giving me thoughtful feedback. Thank you also for reading my thesis and making it better with your comments.

My dissertation advisory committee: David Scadden, Konrad Hochedlinger, and Amy Wagers for your always thoughtful, honest, and helpful feedback. For thinking hard about my data and making me a better scientist.

My thesis advisory committee: Jay Rajagopal, Alex Meissner, and George Murphy. For embarking on the final step of this grad school journey with me and reading my thesis.

Laurie Boyer for being my mentor since my undergrad years and shaping me as a scientist. For believing in me and teaching me to keep going and not give up even in the most difficult times. You are an inspiration.

My sisters: Julie Sneddon for so many moments in and out of the lab, advice about science and life, and laughter. Thank you for being the most caring and thoughtful and always watching out for me. Ching Kao for your wisdom, wittiness, and sincerity. For being a guide and companion through this process and understanding the many me's. Debbie Varela for your lightheartedness, uniqueness, and unconditional love, and for always reminding me how far I've come. Melanie Cornejo for being a super-woman that inspires me every day. Nuri Mouriño for unforgettable moments and conversations and for making time stop, near and far.

José Rivera Feliciano, for making me laugh with the different ways that two stem cells can interact at a bar, for always being positive, for interesting discussions and thoughtful suggestions, and for always knowing a solution to everything. You are an amazing scientist and I'm lucky to call you my friend.

Alice Chen, my roomie for the first half of my grad school years. Thank you for your always-thoughtful advice, your laughter, and your dedication to beautiful science. I look forward to a lifetime of friendship with you.

Bill Anderson, for being the kindest, funniest, and classiest lunch companion. Your advice on teaching, science, life, and Boston knows no match.

Melinda Snitow for showing me how to grow hES cells, make virus, transduce cells, and many techniques that were crucial throughout grad school and for making me obsessive about sterile technique.

Cole Arledge for being the best student I could wish for and teaching me how to be a better mentor. Your dedication, tenacity, and enthusiasm were contagious and you made the lab a great place to work.

Peng Yi , Gosia Borowiak and Barak Blum for being great bay-mates and scientists. Thank you for your advice on how to make my project better.

Justin Annes for your interest and advice on my project and for your help with image analysis.

Fernando Camargo and Mori Mohseni for collaborating with me on the Hippo story.

Chunhui Xie for taking care of my cells while I wrote my thesis. Thanks to you I was able to get (at least some) sleep.

Jenny Ryu for your positive energy. For teaching me how to do Western Blots and for always laughing at my bad jokes.

Christian Honoré and Quinn Peterson, the apoptosis pros, for thoughtful advice for my project.

Felicia Pagliuca, Ornella Barrandon, Sundari Chetty, Jeff Millman, and Christian Schuetz for bringing new energy to the lab. Peter Carolan for interesting discussions and for insightful comments. Claire Bonal for your sincerity and strength. Siniša Hrvatin, my fellow grad student, for your thoughtfulness and always interesting conversations.

Ollie Belmont and George Kenty for allowing the lab to run and facilitating experiments.

Millie Ray for your friendship and advice for science and life and for reminding me of my MIT days when we learned to be scientists together.

Alison Taylor, Monica Markovski, April Cook, and the rest of my BBS friends, for sharing encouragement and fun times during grad school.

My students in SCRB 165, SCRB 10, and SCRB 140 for your enthusiasm, curiosity, and perseverance towards learning stem cell biology.

Past and present Meltonites for thinking big, working hard, and making the Melton Lab a great place to come to every day.

Joy LaVecchio and Giri Buruzula for your amazing help with all things FACS. For being truly dedicated to what you do and a pleasure to work with.

Kelvin Lam for his help with high-throughput image analysis.

René Maehr for his collaboration in the gain-of-function screen.

Saranya Purushothaman for helping with obtaining ORFeome reagents.

Rudolf Jaenisch for introducing me to the world of research and to the fascinating biology of early embryonic development and embryonic stem cells.

Adelaida Sarukhán, for the eye-opening explanations that made me want to become a biologist.

Nono, for inspiring me to become a scientist and for being the example of a true scholar. I miss you every day.

My family for being close despite the distance and helping me become who I am today.

Roxana and Carlos for being an example of perseverance and hard work and for being my family. Your support and love is invaluable.

Alejandro, for awakening the kid in me. Dream big, Niño.

Héctor, for your friendship and companionship since I was 2 years old. Your conviction and passion inspire me every day.

My parents, María Eugenia and Héctor, for inspiring me to go as far as I could see, even if that meant being away from you. For teaching me to always believe in myself and follow my heart. For feeding my creativity and imagination since I was a kid and making education a priority. Thank you for always being proud of me.

Filip for bringing out the best in me and inspiring me to grow further. Thank you for your support and love throughout this journey, for always instilling confidence in me, and for making this finished thesis so pretty. You are my best friend, my inspiration, my rock, my love. I am fortunate to look into your eyes every day.

*To my parents and my brothers,
for supporting me in the pursuit of my dreams
and to Filip, for dreaming with me.*

Chapter 1

Introduction

Embryonic stem (ES) cells are isolated from the inner cell mass of the developing blastocyst embryo. These cells have the remarkable ability to give rise to all the cell types in the embryo proper, a characteristic termed as pluripotency. In addition, ES cells can self-renew – i.e. maintain their own population by giving rise to cells with their same characteristics upon cell division in-vitro. Self-renewal of hES cells allows the expansion of a pluripotent population that can be directed to become a variety of cell types. Therefore, ES cells have important medical potential for cell replacement therapy of multiple diseases. For this reason, understanding the molecular mechanisms that guide cellular decisions to survive, divide, and differentiate will bring us one step closer to harnessing the potential of hES cells for regenerative medicine.

1.1 History of pluripotent cells

1.1.1. Teratocarcinoma and embryonal carcinoma cells

The study of pluripotent cells began in the 1950s with teratocarcinomas and embryonal carcinoma (EC) cells. In seminal work in the 1950s, Stevens discovered tumors arising spontaneously in the testes of the 129 mouse strain at very high incidence (1%). These tumors contained cell types from the three developmental germ layers (ectoderm, mesoderm, and endoderm) [1]. In contrast to teratomas, teratocarcinomas are considered malignant tumors as they contain a population of undifferentiated embryonal carcinoma cells capable of propagating the tumor when transplanted into a secondary mouse recipient [2]. Studies in the late 1960s and 70s then found that teratocarcinomas could also be formed by transplanting pre-gastrulation embryos into the testes or kidney of recipient adult mice [3]. In addition, it was determined that the cell of origin for these tumors were cells in the epiblast [4]. Subsequently, EC cells were adapted to culture. It is thanks to those early studies that the conditions for the later derivation and propagation of mouse ES (mES) cells were established, including the use of fibroblast feeder cells [2, 5].

Human EC cells were derived almost concurrently to mES cells [6]. These cells also served as tools to model early human development and differentiation and were helpful for uncovering many of the molecular markers that are still used to identify pluripotent cells today [7]. However, the conditions in which these lines were derived and subsequently maintained have been found to greatly affect the propensity of different lines –including clonal cell lines from the same parent line – to differentiate into certain

lineages [5]. Interestingly, the conditions for maintaining human EC cells are very different from the conditions used to maintain human ES (hES) cells. These differences, along with the variability of their differentiation, made human EC cells less useful as a model for human differentiation and development.

1.1.2. Mouse and human embryonic stem cell derivation

In 1981, Martin Evans, Matthew Kauffman, and Gail Martin isolated embryonic stem cells from mouse blastocysts for the first time [8, 9]. These cells provided a more accurate model than EC cells to study early developmental decisions at the cellular level. Even though they shared many characteristics with EC cells, an interesting distinction was that they could contribute to the germline when transplanted into recipient blastocysts [10]. ECs only contributed to somatic tissues at a relatively low rate. Germline transmission opened the door to the field of genetic modification and the generation of transgenic mice.

Human ES cells were derived in 1998 by James Thomson [11]. The lack of optimized culture conditions was the main reason why hES cells were not established sooner [5]. It is now clear that the signals that maintain hES cell self-renewal are very different from those used to maintain mouse ES cells, preventing the straightforward translation of knowledge gained with mES cells to hES cells. Nonetheless, under the appropriate conditions, embryonic stem cells from both species can be maintained indefinitely in culture while retaining the ability to give rise to cellular derivatives from the three embryonic germ layers. These two characteristics –self-renewal and pluripotency– have important implications for regenerative

medicine and exciting research is underway to guide the differentiation of these cells for cell replacement therapies of multiple diseases.

1.2 Mouse embryonic stem cell self-renewal

Two signals have been shown to sustain self-renewal in cultured mES cells: Leukemia Inhibitory Factor (LIF) and Bone Morphogenetic Protein (BMP). LIF and BMP maintain mES cell pluripotency by complementary mechanisms that activate self-renewal and inhibit differentiation, respectively [12]. LIF signals via the gp130 cytokine receptor and the LIF receptor to activate JAK kinases, which, in turn, phosphorylate STAT3. Phosphorylated STAT3 forms a homodimer that translocates to the nucleus and activates transcription of target genes that promote self-renewal, including *Klf4*, *Sall4*, and *Jmjd* [13-15]. This signaling is complemented by BMP, a growth factor found in serum, which activates the inhibitor of differentiation (Id) genes, which in turn inhibit neural differentiation in mES cells [12]. Id proteins are helix-loop-helix proteins that act as dominant negative inhibitors of several families of transcriptional regulators, many of these with roles in lineage specification [16, 17]. Basic helix-loop-helix (bHLH) transcription factors are sequestered by Ids. Since Id proteins lack a basic DNA binding domain, they prevent the dimerization of bHLH factors with other tissue-specific partners thus hindering their binding to DNA [18]. Normally, inactivated mouse embryonic fibroblasts (MEFs) serve as a source of LIF and BMP is provided by the addition of serum to the medium. Therefore, when used together, BMP and LIF allow the maintenance of pure mES cell populations in feeder-free and serum-free conditions [12] (**Figure 1-1**). These conditions are advantageous because they are better defined, thus allowing more control of the signals provided to cells, both for maintenance and for directed differentiation efforts.

Autocrine Fibroblast Growth Factor (FGF)-4 signaling in mES cells leads to activation of Extracellular-signal Regulated Kinase (ERK) signaling. It is thought that this signal primes ES cells for differentiation into multiple lineages [19, 20]. To find a stable ground or naïve state for self-renewal, Austin Smith's group used an ERK inhibitor to prevent this priming into lineage commitment. Additionally, Glycogen Synthase Kinase 3 β (GSK3- β) was inhibited to promote mES cell propagation. Together, inhibitors for these two pathways are known as 2i. The culture of mES cells in 2i with or without LIF allows serum independence and is permissive for the culture of refractive ES lines [19-22] and ES lines from previously non-permissive species [23]. Smith proposes that when mES cells are maintained with LIF and BMP, FGF4-ERK signaling is active, and therefore these cells are already in a primed state for differentiation. By inhibiting FGF4-ERK, priming is inhibited, and therefore mES cells are kept in a naïve or ground state of self-renewal [19, 20]. It is thought that changing culture conditions or overexpressing genes with known roles in pluripotency and reprogramming, such as Klf4 [24], can allow the conversion from naïve to primed state and vice versa, but the changes that occur at the molecular level and the role of the pluripotency master regulators is only beginning to be understood [25].

1.3 Human embryonic stem cell self-renewal and survival

1.3.1. *Self-renewal mechanisms in hES cells*

Signaling pathways involved in hES cell self-renewal –or at least their upstream effectors– have been shown to be divergent from the mouse. For instance, LIF signaling does not sustain self-renewal in hES cells. Studies have shown that even though hES cells are molecularly capable of responding to LIF signaling by upregulating STAT3 phosphorylation, this does not contribute to maintenance of self-renewal [26, 27]. This result is not due to phosphorylation levels below an activation threshold, as the expression of a constitutively active form of STAT3 was not sufficient to maintain the undifferentiated hES cell state [26]. BMP also failed to contribute to hES cell self-renewal, and instead caused differentiation of hES into trophectoderm. In addition to morphological changes, this differentiation was accompanied by upregulation of genes involved in trophoblast and placental development [28]. Recently, it was also shown that short-term BMP signaling in hES cells could lead to mesoderm differentiation [29]. Conversely, culturing hES cells in media containing the BMP inhibitor noggin has been shown to result in decreased differentiation [30].

Activin A and Fibroblast Growth Factor (FGF) signaling pathways are now understood to be important for hES cell maintenance. However, it is possible that other pathways contributing to self-renewal remain to be discovered. Activin A has been shown to act synergistically with FGF2 to promote self-renewal [31-34]. Activin A/Nodal/TGF β binding to the Type I and Type II receptors promotes a cross-phosphorylation event that results in the phosphorylation and activation of Smad 2/3.

Activated Smad 2/3 binds to the Smad4 co-Smad and translocates to the nucleus where it interacts with other transcription factors to regulate gene expression [35]. A study by Xu and colleagues found that Smad2/3 binds to the Nanog promoter and promotes transcription of Nanog in hES cells [36]. Xu also showed that FGF contributed to proliferation and supported Nanog expression, but how it interacts with the pluripotency network remains to be determined. Interestingly, this study also uncovered the mechanism for differentiation induction by BMP in hES cells. Smad1/5/8, which are activated in response to BMP can also bind and inhibit the Nanog promoter. Overall, not only do Activin A and FGF induce each other's transcription, but they also seem to act together to counteract the activity of BMP (**Figure 1-1**).

Despite differences in the upstream signaling mechanisms that promote pluripotency, mouse and human ES cells depend on three master regulators of pluripotency: Oct4, Sox2, and Nanog. These transcription factors bind to the promoter region of multiple genes in ES cells [37, 38]. To maintain the ES cell state, they promote the expression of genes important for pluripotency and self-renewal by recruiting the transcriptional machinery. In addition, they inhibit genes that lead to the establishment of differentiation programs by recruiting chromatin-modifying proteins that promote gene repression such as Polycomb group proteins [39, 40].

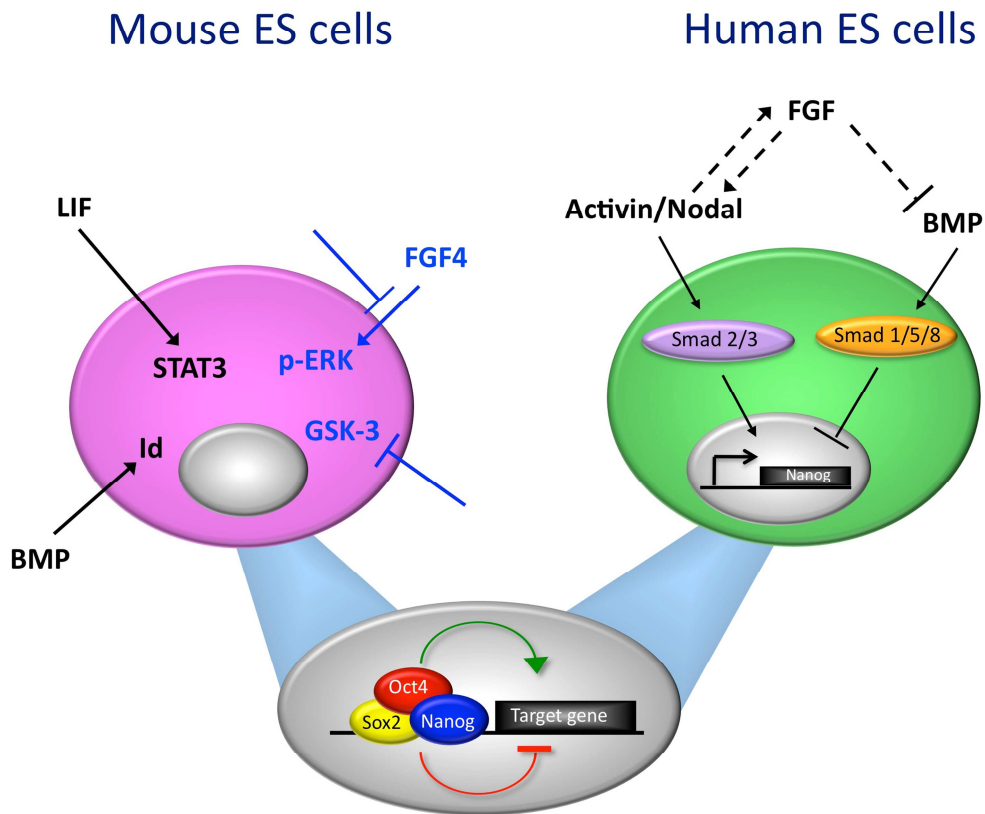


Figure 1-1. Self-renewal mechanisms differ between mouse and human ES cells. Mouse ES cells can be maintained by LIF (acting through STAT3) and BMP (inducing Id genes) in the primed state. In the naïve state (indicated in blue), inhibition of ERK and GSK-3 β allow the maintenance of mES cells in the ground state. Human ES cells are maintained by Activin/TGF β (which act through Smad2/3 and promote Nanog expression) and FGF. FGF and Activin promote each other's expression. Additionally, FGF might participate in the inhibition of BMP through an unknown mechanism. BMP promotes differentiation in hES cells by inhibiting Nanog expression. Oct4, Sox2, and Nanog orchestrate a pluripotency transcriptional program in both species.

1.3.2. Survival mechanisms in hES cells

Besides the upstream signaling that leads to their self-renewal, another distinctive characteristic between human and mouse ES cells is the sensitivity of hES cells to dissociation leading to high levels of cell death. It is not understood why mouse ES cells do not share this sensitivity to dissociation stress, but some have suggested a differential response by cells in different developmental states – mES cells being more ICM-like or “naïve” and hES cells being more epiblast-like or “primed” [41]. Dissociation-induced cell death has been a roadblock to the application of hES cells to methods where clonal cells are needed, including gene manipulation and the study of clonal populations. In 2007 a chemical inhibitor of Rock was shown to increase survival of dissociated hES cells by close to 30-fold [42]. Rock inhibitor (Y-27632) is now commonly used in the maintenance and derivation of hES and iPS cells.

Recently, several studies have allowed us to understand the relation between cell dissociation, cell death, and Rock inhibitor. In an epithelial colony, where cell-to-cell contacts are intact E-cadherin mediates inhibition of Active BCR-Related gene (Abr), and activated Ras-related C3 botulinum toxin substrate 1 (Rac) inhibits Ras homolog gene family members (Rho). Disruption of cell-to-cell contacts lead to the activation of Abr. Abr is both a Rho-GEF, catalyzing the activation of Rho by binding of GTP and a Rac-GAP, inactivating Rac by GTP hydrolysis. Therefore, Rac is inactivated and Rho is activated. Activated Rho leads to the activation of Rho-associated kinase (Rock), which phosphorylates myosin. This leads to myosin hyperactivation, manifested as blebbing of

the cell membrane and preceding caspase activation and cell death in hES cells [41, 43, 44] (**Figure 1-2**).

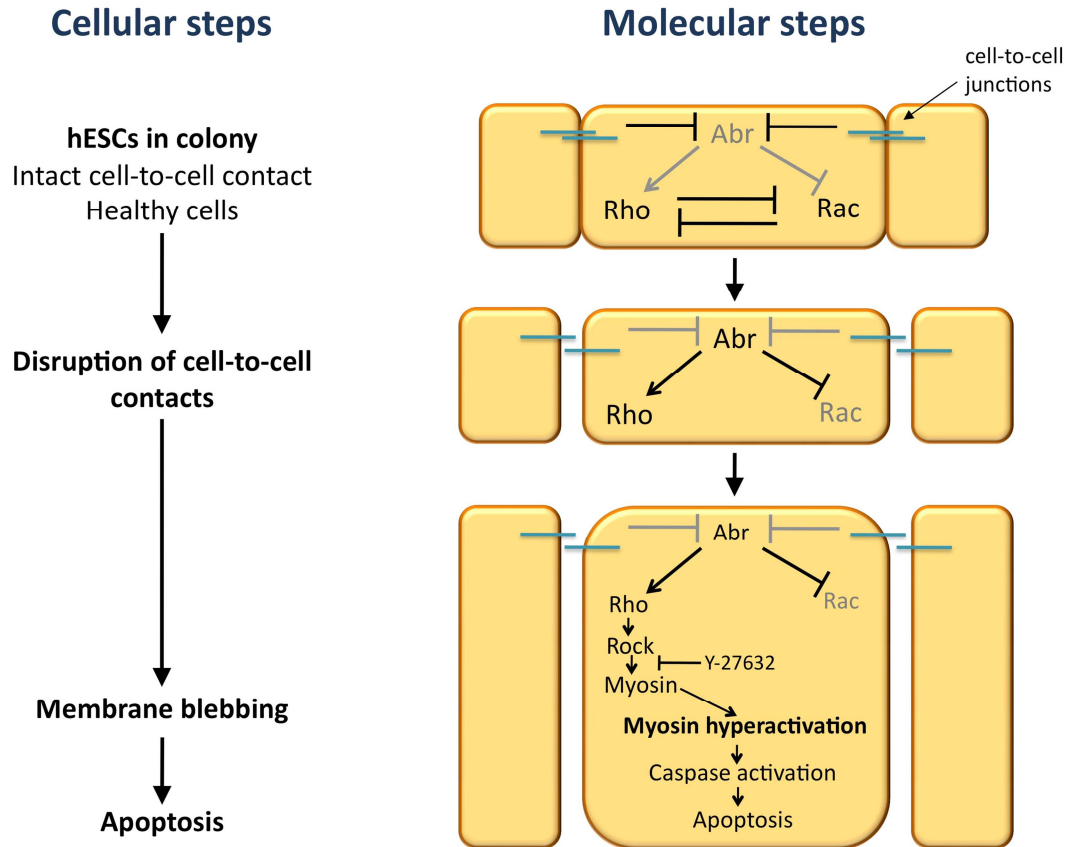


Figure 1-2. Cell dissociation promotes apoptosis in hES cells. E-cadherin-mediated cell-to-cell junctions inhibit Abr. When cell-to-cell junctions are disrupted, Abr activates Rho and inhibits Rac. Rho activates Rock, which phosphorylates myosin, leading to myosin hyperactivation and blebbing). Myosin hyperactivation sets off caspase-mediated apoptosis. (Figure adapted from [41])

1.4 Induced pluripotent stem cells

Work in cloning and somatic cell nuclear transfer showed that the nucleus of an adult cell retained the potential to give rise to an entire organism when placed in an enucleated oocyte [45]. This experiment revealed that the genome of a cell was not irreversibly changed during development. For several decades, research focused on finding the combination of factors that made this possible in the oocyte.

A breakthrough by Takahashi and Yamanaka in 2006 [46] found that co-expression of Oct4, Sox2, Klf4, and c-Myc –four transcription factors known to be important in pluripotent cells– could reprogram an adult fibroblast into an embryonic stem cell state. These induced pluripotent stem (iPS) cells were assessed for their pluripotent ability by the pluripotent marker expression, teratoma formation, and contribution to developing tissues in chimeric mice. This combination of factors was found to also reprogram human fibroblasts [47-49]. iPS cells allow the derivation of patient-specific pluripotent cells, therefore circumventing the biological and ethical roadblocks of producing them by other means. Before the discovery of iPS cells, the alternative for obtaining patient-specific pluripotent cells was somatic cell nuclear transfer (SCNT). SCNT is the technology used to clone Dolly the sheep and involves injecting a somatic cell nucleus into an enucleated oocyte to obtain blastocysts [45]. These blastocysts can then be used to derive ES lines, or as in the case of Dolly, be implanted into the uterus of a recipient mother for organism development. Besides being very inefficient [50, 51], SCNT has obvious shortcomings in humans, including the

accessibility of oocytes for nuclear injection [52] and the fact that many consider it “too close a step” in cloning humans.

The factors used to reprogram fibroblasts, have since been shown to reprogram a variety of adult cell types to pluripotency [53-55]. Additionally, much progress and numerous technological advances have happened since to improve reprogramming efficiency from an initial 0.001% to up to 4.4% [56]. Currently, intense research is in progress to determine whether ES cells and iPS cells are functionally equivalent. Some have suggested that the transcriptional and epigenetic differences between these populations have to do with their different derivation histories [56]. Nevertheless, they are both valuable pluripotent populations with the potential to make the goals of regenerative medicine a reality.

1.5 Genetic screening approaches to understanding self-renewal

Screening provides a broad, high-throughput approach to discovering genes involved in a particular biological process. A few studies have undertaken a genetic screening approach to discover genes involved in the maintenance of self-renewal in hES cells. These studies, however, have focused on a loss-of-function approach, where the use of siRNA has allowed for the discovery of genes necessary for the hES cell state. Of note is a study by Chia and colleagues [57]. This study used a hES cell line with a GFP reporter under the control of the Oct4 promoter. This study surveyed over 21,000 shRNAs in a primary assay. 200 candidates were selected for further verification based on their ability to downregulate GFP levels and their gene ontology categories. PR-domain-containing 14 (PRDM14) was identified as a co-transcriptional regulator with a role in regulating the transcription of Oct4 and the ability to increase the efficiency of reprogramming. This study serves as a proof-of-principle for the use of loss-of-function genetic screens for gene discovery in hES cells.

Despite the advantages provided by screening approaches, no gain-of-function studies have been reported for hES cells. Interestingly, Nanog was discovered using a small-scale gain-of-function screen in mouse ES cells [58]. In this approach a cDNA library was produced from mouse ES cells to look for genes that could overcome ablation of the LIF-receptor. The reader might wonder why genetic gain-of-function screens have not been a popular approach for hES cells. Perhaps creating an assay for sufficiency in hES cells has been a roadblock for many. This could be explained by the

observation that many genes, including Oct4 and Sox2, need to be expressed at carefully defined levels to maintain pluripotency and deviation from these levels results in differentiation [59-62]. An alternative explanation is the availability of sophisticated reporters in mES cells, making them a more attractive cell type to use for screening purposes. Researchers might prefer to verify genes discovered in mES cells instead of generating a screening strategy for hES cells. As this chapter outlined previously, there are, however, important differences between mouse and human. It is clear that there is much to learn about self-renewal and pluripotency in hES cells. Screening strategies are an efficient way to approach the puzzle, but require careful optimization of conditions and controls to expedite gene discovery.

1.6 Some current challenges and questions in the field

Human ES and iPS cells represent a unique opportunity to attain the goal of regenerative medicine. If we consider the ES cell state as an equilibrium of mechanisms promoting self-renewal, inhibiting differentiation, and promoting proliferation and survival, we have only started to understand the mechanistic details of these processes and how they relate to each other. A deeper understanding of these mechanisms will allow us to better define the pluripotent state and perhaps establish whether there are commonalities in the maintenance and expansion of progenitor cell pools in vitro.

Despite rapid progress in this field, central questions remain in our basic understanding of hES cell biology. For instance, what other pathways, besides Activin/TGF β , are necessary for self-renewal? How are these pathways maintaining the pluripotency network in hES cells and which combination of factors is sufficient for hES cell maintenance? Understanding the pathways required to maintain hES cell pluripotency will allow culturing of hES cells in defined conditions. Defined conditions have many advantages, including the ability to more efficiently direct self-renewal and differentiation, and the independence from animal-derived components. The field has progressively shifted towards culture of hES cells in the absence of MEFs and serum. However, it is worth noting that the concentration of FGF currently used in conditioned media is extremely high (10 fold higher than the concentration used for hES cell maintenance on MEFs) [30, 63], perhaps because the molecular foundation of hES cell self-renewal is not entirely understood. The consequences of such high concentrations are unknown, for instance in terms of possible cross-reactivity with other pathways or

in the generation of epigenetic changes that affect the differentiation potential of pluripotent cells. Additionally, the connections of FGF with the pluripotency transcription factors are not understood.

The molecular underpinnings of many differences between mouse and human ES cells are not well understood. Given that mouse ES cells, and cells in the naïve state are not sensitive to dissociation, we consider that gaining a better understanding of the mechanisms that change between the naïve and primed state would allow us to understand the causes leading to changes in morphology and an increased sensitivity to dissociation.

1.7 Overview of the dissertation

In this dissertation, we aim to gain a better understanding into the mechanisms behind hES cell maintenance. Chapter 2 describes a gain-of-function screen using a human open-reading-frame (ORFeome) library to find genes that can overcome inhibition of TGF β signaling. It also describes a verification assay for candidates obtained from that primary screen and the identification of Vestigial-like 4 (Vgll4) a co-transcriptional regulator that had not been described previously in hES cells.

Chapter 3 focuses on characterizing the role of Vgll4 in promoting survival of hES cells. We find that Vgll4 protects hES cells from dissociation stress and allows a higher survival rate. This is possibly due to the inhibition of caspase activity and perhaps interaction with the Rock pathway.

Chapter 4 covers other mechanisms that were explored in the effort to characterize the function of Vgll4. We present data that suggests a complex mechanism of action for this gene and the possible involvement of other pathways and processes in its observed phenotype. Finally, Chapter 5 covers some overall conclusions and discusses insights as well as future opportunities for this work.

References

- [1] L. C. Stevens and K. P. Hummel, "A description of spontaneous congenital testicular teratomas in strain 129 mice," *J Natl Cancer Inst*, vol. 18, pp. 719-47, May 1957.
- [2] I. Chambers and A. Smith, "Self-renewal of teratocarcinoma and embryonic stem cells," *Oncogene*, vol. 23, pp. 7150-7160, 2004.
- [3] L. C. Stevens, "The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos," *Developmental Biology*, vol. 21, pp. 364-382, 1970.
- [4] S. B. Diwan and L. C. Stevens, "Development of teratomas from the ectoderm of mouse egg cylinders," *J Natl Cancer Inst*, vol. 57, pp. 937-42, Oct 1976.
- [5] J. Yu and J. A. Thomson, "Pluripotent stem cell lines," *Genes Dev*, vol. 22, pp. 1987-97, Aug 1 2008.
- [6] B. Hogan, M. Fellous, P. Avner, and F. Jacob, "Isolation of a human teratoma cell line which expresses F9 antigen," *Nature*, vol. 270, pp. 515-8, Dec 8 1977.
- [7] P. W. Andrews, P. N. Goodfellow, L. H. Shevinsky, D. L. Bronson, and B. B. Knowles, "Cell-surface antigens of a clonal human embryonal carcinoma cell line: morphological and antigenic differentiation in culture," *Int J Cancer*, vol. 29, pp. 523-31, May 15 1982.
- [8] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, pp. 154-6, Jul 9 1981.
- [9] G. R. Martin, "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells," *Proc Natl Acad Sci U S A*, vol. 78, pp. 7634-8, Dec 1981.
- [10] A. Bradley, M. Evans, M. H. Kaufman, and E. Robertson, "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines," *Nature*, vol. 309, pp. 255-6, May 17-23 1984.
- [11] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall, and J. M. Jones, "Embryonic Stem Cell Lines Derived from Human Blastocysts," *Science*, vol. 282, pp. 1145-1147, November 6, 1998 1998.
- [12] Q.-L. Ying, J. Nichols, I. Chambers, and A. Smith, "BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3," *Cell*, vol. 115, pp. 281-292, 2003.
- [13] H. Niwa, T. Burdon, I. Chambers, and A. Smith, "Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3," *Genes Dev.*, vol. 12, pp. 2048-2060, July 1, 1998 1998.
- [14] P.-Y. Bourillot, I. Aksoy, V. Schreiber, F. Wianny, H. Schulz, O. Hummel, N. Hubner, and P. Savatier, "Novel STAT3 Target Genes Exert Distinct Roles in the

- Inhibition of Mesoderm and Endoderm Differentiation in Cooperation with Nanog," *Stem Cells*, vol. 27, pp. 1760-1771, 2009.
- [15] B. L. Kidder, J. Yang, and S. Palmer, "Stat3 and c-Myc Genome-Wide Promoter Occupancy in Embryonic Stem Cells," *PLoS One*, vol. 3, p. e3932, 2008.
 - [16] R. Benezra, R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub, "The protein Id: A negative regulator of helix-loop-helix DNA binding proteins," *Cell*, vol. 61, pp. 49-59, 1990.
 - [17] D. Lyden, A. Z. Young, D. Zagzag, W. Yan, W. Gerald, R. O'Reilly, B. L. Bader, R. O. Hynes, Y. Zhuang, K. Manova, and R. Benezra, "Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts," *Nature*, vol. 401, pp. 670-677, 1999.
 - [18] P. J. O'Toole, T. Inoue, L. Emerson, I. E. G. Morrison, A. R. Mackie, R. J. Cherry, and J. D. Norton, "Id Proteins Negatively Regulate Basic Helix-Loop-Helix Transcription Factor Function by Disrupting Subnuclear Compartmentalization," *J. Biol. Chem.*, vol. 278, pp. 45770-45776, November 14, 2003 2003.
 - [19] J. Silva and A. Smith, "Capturing Pluripotency," *Cell*, vol. 132, pp. 532-536, 2008.
 - [20] Q.-L. Ying, J. Wray, J. Nichols, L. Battle-Morera, B. Doble, J. Woodgett, P. Cohen, and A. Smith, "The ground state of embryonic stem cell self-renewal," *Nature*, vol. 453, pp. 519-523, 22 May 2008 2008.
 - [21] J. Nichols and A. Smith, "Naive and Primed Pluripotent States," *Cell Stem Cell*, vol. 4, pp. 487-492, 2009.
 - [22] J. Hanna, S. Markoulaki, M. Mitalipova, A. W. Cheng, J. P. Cassady, J. Staerk, B. W. Carey, C. J. Lengner, R. Foreman, J. Love, Q. Gao, J. Kim, and R. Jaenisch, "Metastable pluripotent states in NOD-mouse-derived ESCs," *Cell Stem Cell*, vol. 4, pp. 513-24, Jun 5 2009.
 - [23] M. Buehr, S. Meek, K. Blair, J. Yang, J. Ure, J. Silva, R. McLay, J. Hall, Q.-L. Ying, and A. Smith, "Capture of Authentic Embryonic Stem Cells from Rat Blastocysts," *Cell*, vol. 135, pp. 1287-1298, 2008.
 - [24] J. Hanna, A. W. Cheng, K. Saha, J. Kim, C. J. Lengner, F. Soldner, J. P. Cassady, J. Muffat, B. W. Carey, and R. Jaenisch, "Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs," *Proc Natl Acad Sci U S A*, vol. 107, pp. 9222-7, May 18 2010.
 - [25] Y. Miyanari and M.-E. Torres-Padilla, "Control of ground-state pluripotency by allelic regulation of Nanog," *Nature*, vol. 483, pp. 470-473, 2012.
 - [26] L. Daheron, S. L. Opitz, H. Zaehres, W. M. Lensch, P. W. Andrews, J. Itskovitz-Eldor, and G. Q. Daley, "LIF/STAT3 Signaling Fails to Maintain Self-Renewal of Human Embryonic Stem Cells," *Stem Cells*, vol. 22, pp. 770-778, September 1, 2004 2004.

- [27] R. K. Humphrey, G. M. Beattie, A. D. Lopez, N. Bucay, C. C. King, M. T. Firpo, S. Rose-John, and A. Hayek, "Maintenance of Pluripotency in Human Embryonic Stem Cells Is STAT3 Independent," *Stem Cells*, vol. 22, pp. 522-530, July 1, 2004 2004.
- [28] R.-H. Xu, X. Chen, D. S. Li, R. Li, G. C. Addicks, C. Glennon, T. P. Zwaka, and J. A. Thomson, "BMP4 initiates human embryonic stem cell differentiation to trophoblast," *Nat Biotech*, vol. 20, pp. 1261-1264, 2002.
- [29] P. Zhang, J. Li, Z. Tan, C. Wang, T. Liu, L. Chen, J. Yong, W. Jiang, X. Sun, L. Du, M. Ding, and H. Deng, "Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells," *Blood*, pp. blood-2007-02-074120, November 27, 2007 2007.
- [30] R.-H. Xu, R. M. Peck, D. S. Li, X. Feng, T. Ludwig, and J. A. Thomson, "Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells," *Nat Meth*, vol. 2, pp. 185-190, 2005.
- [31] G. M. Beattie, A. D. Lopez, N. Bucay, A. Hinton, M. T. Firpo, C. C. King, and A. Hayek, "Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers," *Stem Cells*, vol. 23, pp. 489-495, April 1, 2005 2005.
- [32] B. Greber, H. Lehrach, and J. Adjaye, "Fibroblast Growth Factor 2 Modulates Transforming Growth Factor {beta} Signaling in Mouse Embryonic Fibroblasts and Human ESCs (hESCs) to Support hESC Self-Renewal," *Stem Cells*, vol. 25, pp. 455-464, February 1, 2007 2007.
- [33] D. James, A. J. Levine, D. Besser, and A. Hemmati-Brivanlou, "TGF{beta}/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells," *Development*, vol. 132, pp. 1273-1282, March 15, 2005 2005.
- [34] L. Xiao, X. Yuan, and S. J. Sharkis, "Activin A Maintains Self-Renewal and Regulates Fibroblast Growth Factor, Wnt, and Bone Morphogenic Protein Pathways in Human Embryonic Stem Cells," *Stem Cells*, vol. 24, pp. 1476-1486, June 1, 2006 2006.
- [35] J. Massague, S. W. Blain, and R. S. Lo, "TGF[beta] Signaling in Growth Control, Cancer, and Heritable Disorders," *Cell*, vol. 103, pp. 295-309, 2000.
- [36] R. H. Xu, T. L. Sampsell-Barron, F. Gu, S. Root, R. M. Peck, G. Pan, J. Yu, J. Antosiewicz-Bourget, S. Tian, R. Stewart, and J. A. Thomson, "NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs," *Cell Stem Cell*, vol. 3, pp. 196-206, Aug 7 2008.
- [37] L. A. Boyer, T. I. Lee, M. F. Cole, S. E. Johnstone, S. S. Levine, J. P. Zucker, M. G. Guenther, R. M. Kumar, H. L. Murray, R. G. Jenner, D. K. Gifford, D. A. Melton, R. Jaenisch, and R. A. Young, "Core transcriptional regulatory circuitry in human embryonic stem cells," *Cell*, vol. 122, pp. 947-56, Sep 23 2005.
- [38] L. A. Boyer, D. Mathur, and R. Jaenisch, "Molecular control of pluripotency," *Curr Opin Genet Dev*, vol. 16, pp. 455-62, Oct 2006.

- [39] L. A. Boyer, K. Plath, J. Zeitlinger, T. Brambrink, L. A. Medeiros, T. I. Lee, S. S. Levine, M. Wernig, A. Tajonar, M. K. Ray, G. W. Bell, A. P. Otte, M. Vidal, D. K. Gifford, R. A. Young, and R. Jaenisch, "Polycomb complexes repress developmental regulators in murine embryonic stem cells," *Nature*, vol. 441, pp. 349-353, 2006.
- [40] T. I. Lee, R. G. Jenner, L. A. Boyer, M. G. Guenther, S. S. Levine, R. M. Kumar, B. Chevalier, S. E. Johnstone, M. F. Cole, K. Isono, H. Koseki, T. Fuchikami, K. Abe, H. L. Murray, J. P. Zucker, B. Yuan, G. W. Bell, E. Herbolsheimer, N. M. Hannett, K. Sun, D. T. Odom, A. P. Otte, T. L. Volkert, D. P. Bartel, D. A. Melton, D. K. Gifford, R. Jaenisch, and R. A. Young, "Control of developmental regulators by Polycomb in human embryonic stem cells," *Cell*, vol. 125, pp. 301-13, Apr 21 2006.
- [41] M. Ohgushi and Y. Sasai, "Lonely death dance of human pluripotent stem cells: ROCKing between metastable cell states," *Trends in Cell Biology*, vol. 21, pp. 274-282, 2011.
- [42] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J. B. Takahashi, S. Nishikawa, K. Muguruma, and Y. Sasai, "A ROCK inhibitor permits survival of dissociated human embryonic stem cells," *Nat Biotechnol*, vol. 25, pp. 681-6, Jun 2007.
- [43] G. Chen, Z. Hou, D. R. Gulbranson, and J. A. Thomson, "Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells," *Cell Stem Cell*, vol. 7, pp. 240-8, Aug 6 2010.
- [44] M. Ohgushi, M. Matsumura, M. Eiraku, K. Murakami, T. Aramaki, A. Nishiyama, K. Muguruma, T. Nakano, H. Suga, M. Ueno, T. Ishizaki, H. Suemori, S. Narumiya, H. Niwa, and Y. Sasai, "Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells," *Cell Stem Cell*, vol. 7, pp. 225-39, Aug 6 2010.
- [45] K. H. Campbell, J. McWhir, W. A. Ritchie, and I. Wilmut, "Sheep cloned by nuclear transfer from a cultured cell line," *Nature*, vol. 380, pp. 64-6, Mar 7 1996.
- [46] K. Takahashi and S. Yamanaka, "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors," *Cell*, vol. 126, pp. 663-676, 2006.
- [47] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, and J. A. Thomson, "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, pp. 1917-20, Dec 21 2007.
- [48] I. H. Park, R. Zhao, J. A. West, A. Yabuuchi, H. Huo, T. A. Ince, P. H. Lerou, M. W. Lensch, and G. Q. Daley, "Reprogramming of human somatic cells to pluripotency with defined factors," *Nature*, vol. 451, pp. 141-6, Jan 10 2008.
- [49] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, pp. 861-72, Nov 30 2007.

- [50] K. Hochedlinger and R. Jaenisch, "Nuclear reprogramming and pluripotency," *Nature*, vol. 441, pp. 1061-7, Jun 29 2006.
- [51] D. Egli, A. E. Chen, G. Saphier, J. Ichida, C. Fitzgerald, K. J. Go, N. Acevedo, J. Patel, M. Baetscher, W. G. Kearns, R. Goland, R. L. Leibel, D. A. Melton, and K. Eggan, "Reprogramming within hours following nuclear transfer into mouse but not human zygotes," *Nat Commun*, vol. 2, p. 488, 2011.
- [52] D. Egli, A. E. Chen, G. Saphier, D. Powers, M. Alper, K. Katz, B. Berger, R. Goland, R. L. Leibel, D. A. Melton, and K. Eggan, "Impracticality of Egg Donor Recruitment in the Absence of Compensation," *Cell Stem Cell*, vol. 9, pp. 293-294, 2011.
- [53] M. Stadtfeld, K. Brennand, and K. Hochedlinger, "Reprogramming of pancreatic beta cells into induced pluripotent stem cells," *Curr Biol*, vol. 18, pp. 890-4, Jun 24 2008.
- [54] S. Eminli, J. Utikal, K. Arnold, R. Jaenisch, and K. Hochedlinger, "Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression," *Stem Cells*, vol. 26, pp. 2467-74, Oct 2008.
- [55] J. Hanna, S. Markoulaki, P. Schorderet, B. W. Carey, C. Beard, M. Wernig, M. P. Creighton, E. J. Steine, J. P. Cassady, R. Foreman, C. J. Lengner, J. A. Dausman, and R. Jaenisch, "Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency," *Cell*, vol. 133, pp. 250-64, Apr 18 2008.
- [56] D. A. Robinton and G. Q. Daley, "The promise of induced pluripotent stem cells in research and therapy," *Nature*, vol. 481, pp. 295-305.
- [57] N. Y. Chia, Y. S. Chan, B. Feng, X. Lu, Y. L. Orlov, D. Moreau, P. Kumar, L. Yang, J. Jiang, M. S. Lau, M. Huss, B. S. Soh, P. Kraus, P. Li, T. Lufkin, B. Lim, N. D. Clarke, F. Bard, and H. H. Ng, "A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity," *Nature*, vol. 468, pp. 316-20, Nov 11 2010.
- [58] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, and A. Smith, "Functional Expression Cloning of Nanog, a Pluripotency Sustaining Factor in Embryonic Stem Cells," *Cell*, vol. 113, pp. 643-655, 2003.
- [59] B. Boer, J. Kopp, S. Mallanna, M. Desler, H. Chakravarthy, P. J. Wilder, C. Bernadt, and A. Rizzino, "Elevating the levels of Sox2 in embryonal carcinoma cells and embryonic stem cells inhibits the expression of Sox2:Oct-3/4 target genes," *Nucleic Acids Research*, vol. 35, pp. 1773-1786, March 1, 2007 2007.
- [60] J. L. Kopp, B. D. Ormsbee, M. Desler, and A. Rizzino, "Small Increases in the Level of Sox2 Trigger the Differentiation of Mouse Embryonic Stem Cells," *Stem Cells*, vol. 26, pp. 903-911, 2008.
- [61] H. Niwa, J.-i. Miyazaki, and A. G. Smith, "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells," *Nat Genet*, vol. 24, pp. 372-376, 2000.
- [62] R. T. Rodriguez, J. M. Velkey, C. Lutzko, R. Seerke, D. B. Kohn, K. S. O'Shea, and M. T. Firpo, "Manipulation of OCT4 Levels in Human Embryonic Stem Cells Results in Induction of Differential Cell Types," *Experimental Biology and Medicine*, vol. 232, pp. 1368-1380, November 1, 2007 2007.

- [63] T. E. Ludwig, M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. A. Daigh, K. R. Conard, M. S. Piekarczyk, R. A. Llanas, and J. A. Thomson, "Derivation of human embryonic stem cells in defined conditions," *Nat Biotech*, vol. 24, pp. 185-187, 2006.

Chapter 2

Genetic gain-of-function screen for self-renewal genes

2.1 Introduction

Pluripotency is the ability of cells such as those in the inner cell mass of the developing blastocyst to contribute to cell derivatives of all germ layers during differentiation. Cells that maintain this unique biological characteristic have been adapted to culture in the form of ES cells and iPS cells, representing fascinating therapeutic potential for multiple diseases. Despite progress in recent years, the molecular mechanisms that maintain the pluripotent state in human embryonic stem (hES) cells, remains poorly understood. This poses a challenge for the maintenance and directed differentiation of these cells into clinically relevant populations. We decided to take a broad approach to address the problem of finding new genes involved in

maintaining the pluripotent state. For this purpose we conducted a biological gain-of-function screen to identify genes that contribute to this process. This chapter will provide a brief description of the library used for this approach, followed by the identification of screening conditions and controls. Finally, this chapter will describe the primary and secondary assays used to discover candidate genes.

The goal of the gain-of-function screen is to find genes that maintain hES cell pluripotency under differentiation conditions. We reason that if a gene is able to prevent a cell from differentiating while allowing it to retain an ES cell phenotype, this gene could be part of the pluripotency maintenance network (**Figure 2-1**). A similar approach was used previously to discover Nanog in mES cells [1]. In this study, LIF signaling –one of the signals that maintains mES cell self renewal (please refer to Chapter 1 for more details)– was ablated by mutation of the LIF-receptor. A cDNA library for genes expressed in mES cells was then used to find genes that could maintain mES cell pluripotency in the absence of irradiated mouse embryonic fibroblasts (MEFs) and LIF.

Dr. René Maehr in our lab led an effort to generate a human open reading frame (ORF)-ome library in lentiviral vectors optimized for expression in mammalian cells. To this end, a human ORFeome library from the Center for Cancer Systems Biology Human ORFeome Collection (horfdb.dfci.harvard.edu) was subcloned into a pHAGE vector [2, 3] adapted for Gateway cloning by the addition of attR sites.

This first version of the ORFeome contains 8,076 ORFs, representing 7,263 genes. All ORFs are tagged with Hemagglutinin (HA) on the C-terminus. The pHAGE vector contains an EF1 α promoter that drives the ORF in question. The EF1 α promoter has

been shown to drive strong expression in hES cells and multiple differentiated cells [4]. In addition, a PGK promoter drives expression of the puromycin-resistance gene, allowing for the selection of a homogeneous population of transduced cells (**Figure 2-2**).

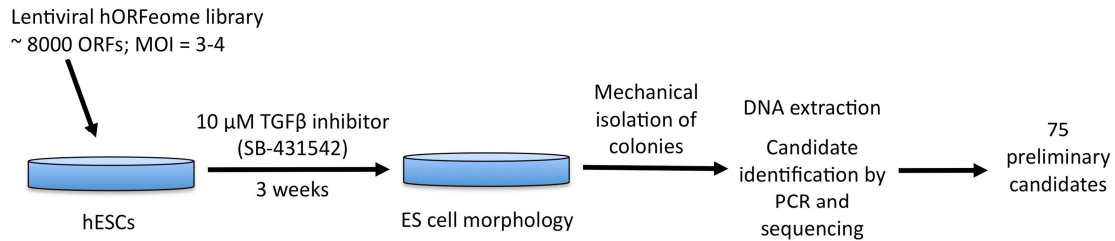


Figure 2-1. A gain-of-function strategy to discover novel genes involved in the maintenance of hES cells. HUES6 cells transduced with lentiviruses carrying a hORFeome library containing approximately 8000 genes (Table S1) were subjected to three weeks of treatment with 10μM TGFβ inhibitor (TGFβi). Colonies with characteristic ES cell morphology after such treatment were picked and their DNA was sequenced to identify 75 preliminary candidates

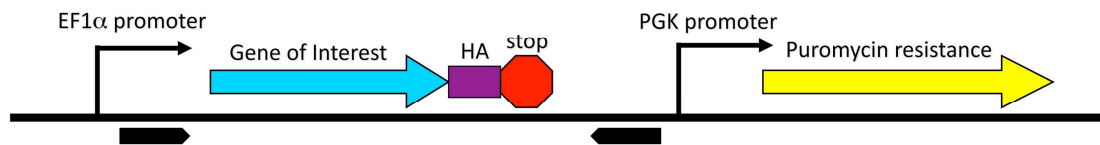


Figure 2-2. A Gateway-adapted pHAGE vector to efficiently promote gene expression in hES cells. The hORFeome library is contained in a lentiviral vector. The gene of interest is driven by an EF1α promoter and the ORF is fused to a hemagglutinin (HA) tag. In addition, this lentiviral vector carries a puromycin resistance gene driven by a PGK promoter. Primer binding sites are indicated by black block arrows under the construct.

2.2 Primary screen

2.2.1. *Experimental Design*

A. Establishment of permissive conditions

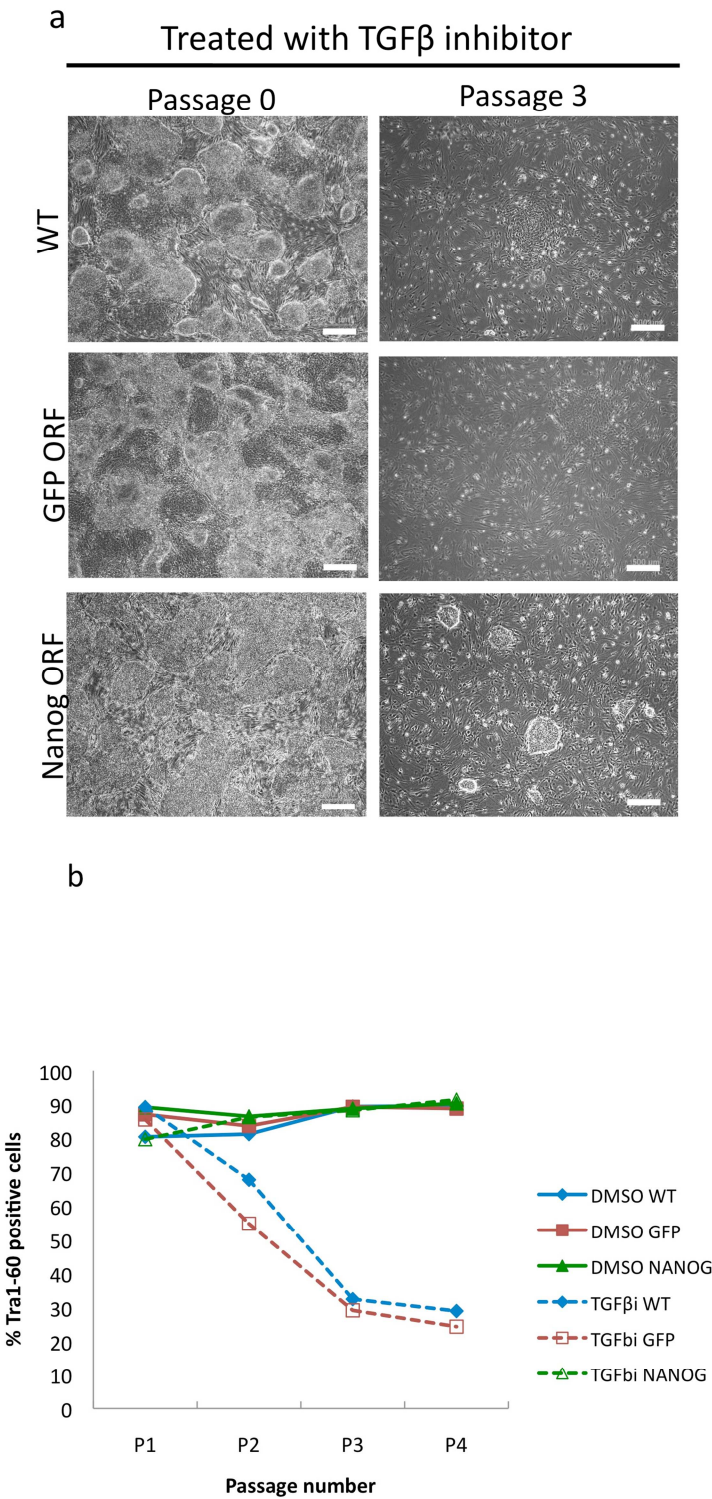
The choice of appropriate screening conditions allows for the discovery of candidates involved in a particular process of interest. We chose to utilize chemical inhibitors for signaling pathways important in the maintenance of hES cell pluripotency. We reasoned that such conditions would allow us to uncover genes with a role in the maintenance of the pluripotent state in hES cells.

The TGF β signaling pathway has been shown to be necessary for the maintenance of pluripotency in hES cells[5-8]. Subsequent studies have shown that Activin/Nodal signaling enhances Nanog expression by binding of Smad 2/3 to its promoter[9]. We therefore predicted that inhibition of this pathway would drive hES cells out of the pluripotent state and towards differentiation.

SB-431542 is a specific inhibitor of the TGF β Type I receptors ALK4, 5, and 7[10]. Based on previously published studies [6, 7], we used a concentration of 10 μ M to disrupt TGF β /Activin/Nodal signaling in hES cells. Treatment of cells with 10 μ M SB-431542 for three passages, or approximately three weeks, caused a loss of hESC morphology and pluripotency marker expression as assessed by microscopy and FACS analysis (**Figure 2-3**).

Figure 2-3. Inhibition of TGF β signaling results in loss of hES cell colony morphology and pluripotency marker expression. Cells transduced with a Nanog ORF were used as a positive control, cells transduced with a GFP ORF and wildtype cells were used as negative controls. a) Negative controls lost the characteristic hESC morphology after three weeks or 3 passages of SB-431542 (TGF β i) treatment (Bars = 500 μ m). b) Cells transduced with GFP and treated with TGF β i for three weeks downregulate Tra1-60 and SSEA-3 (not shown). However, cells transduced with Nanog under this treatment retain expression of these markers.

Figure 2-3 (Continued)



Having established screening controls, we next established positive and negative controls for the screen. Since our screening method is a genetic gain-of-function approach, it was necessary to have a positive control to correctly establish our screening conditions and to efficiently evaluate candidates. Nanog is unique among the core transcription factors in hES cells in that its overexpression prevents lineage specification and maintains cells in a pluripotent state even in the presence of differentiation conditions [1, 11]. Oct4 and Sox2, the other members of the core pluripotency machinery maintain pluripotency at a specific concentration; an increase or decrease in their levels leads to differentiation [12-14]. We therefore chose Nanog overexpression as a positive control for pluripotency maintenance.

GFP was chosen as a negative control. In addition to its lack of effect in pluripotency, it also served as a useful transfection and transduction control in our experiments. In follow up experiments, Tubulin was used as a negative control instead of GFP. This substitution was made since the absence of fluorescence facilitated assays where the GFP channel was needed, for example in FACS and immunocytochemistry.

B. Library transduction

For our screen, we transduced HUES6 hES cells with a pool of genes encompassing version 1.1 of the ORFeome [15]. Lentivirus carrying the ORFeome library was concentrated and titrated by Dr. Maehr. We transduced approximately cells by incubating cells and virus in a low-attachment dish for 2 hours and subsequently plating onto MEFs as a feeder layer. This methodology ensured a low transduction rate of MEFs to maximize the proportion of transduced hES cells. 2×10^6 hES cells were

transduced with 7×10^6 viral particles resulting in an MOI of 3 or 4. Based on these numbers, we estimate that our library was covered close to 1000 times, ensuring adequate coverage.

C. Candidate identification

Following transduction, cells were incubated in hES medium for 48 hours before selecting with $2 \mu\text{g/ml}$ of puromycin. This incubation period ensured enough time for cell attachment, viral integration, and viral gene expression to begin. The GFP control allowed us to assess EF1 α promoter in transduced cells. Additionally, by following the proportion of GFP-positive cells throughout selection, we were also able to assess PGK promoter activity (**Figure 2-4**). Following puromycin selection cells were treated with SB-431542 for three weeks. After this treatment, the colonies that retained hES cell morphology were isolated manually and lysed to obtain genomic DNA. Primers surrounding the ORF region were used for PCR. Gel-purified PCR products were then sequenced and the ORF was identified using BLAST.

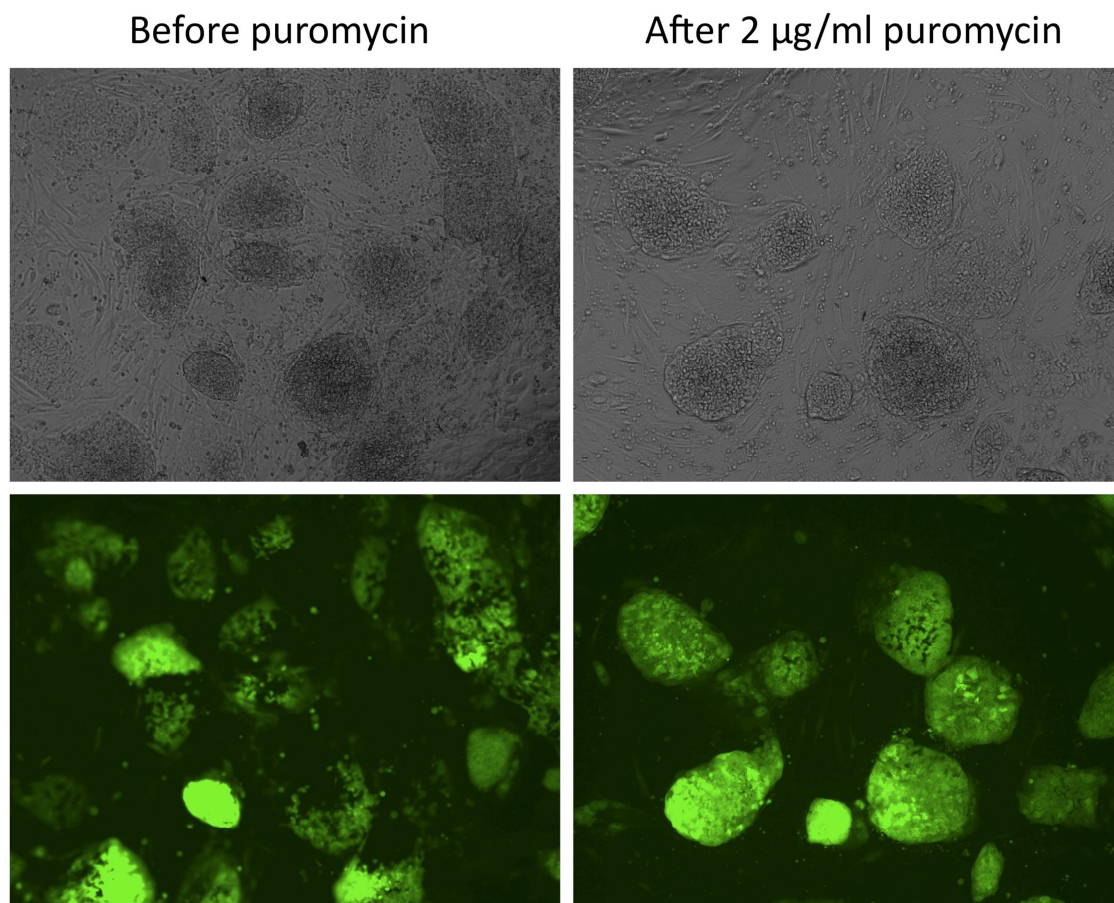


Figure 2-4. EF1 α and PGK promoters drive efficient expression in hESCs. HUES6 cells were transduced with EF1 α -GFP. Before puromycin selection cells that were not transduced are visible. After selecting with 2 $\mu\text{g/ml}$ of puromycin for 2 days, a homogenous population of GFP-expressing cells is observed, indicating efficient activity of both promoters in hES cells.

2.2.2.Results

We identified 75 open reading frames (**Table 2-1**) that were able to maintain hES cell colony morphology under the differentiation conditions that we used for our screen. None of these genes had been previously implicated in pluripotency and they belong to diverse gene ontology groups.

Interestingly, Vestigial-like 4 (Vgll4) was identified 3 independent times, an event with a probability of 1×10^{-6} . All other candidates were identified only once.

Table 2-1 - Candidates from primary ORFeome screen

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
ABHD12B	8766	145447	BC034603	345	11041	C05	Homo sapiens chromosome 14 open reading frame 29	Yes
ABHD12B	56102	145447	BC107142	858	51018	D11	Homo sapiens abhydrolase domain containing 12B, mRNA (cDNA clone MGC:129927 IMAGE:40030549), complete cds.	Yes
ACBD7	2209	414149	BC029526	267	11040	E07	Homo sapiens	Yes
AKR1C3	5842	8644	BC001479	972	11007	E07	Homo sapiens aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	No
AKR1C3	7423	8644	BC019230	972	11054	E06	Homo sapiens aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	No
AKR1C4	13082	1109	BC020744	972	31020	G08	Homo sapiens aldo-keto reductase family 1, member C4 (chlorocone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)	Yes
ARL4A	3861	10124	BC001111, BC003027	603	11029	A10	Homo sapiens ADP-ribosylation factor-like 4A, transcript variant 2	No
ARL6	1857	84100	BC024239	561	11074	H11	Homo sapiens ADP-ribosylation factor-like 6	Yes
C11orf51	3923	25906	BC005156	366	11073	H05	Homo sapiens DKFZP564M082 protein	Yes
C11orf51	5557	25906	BC005393	366	11041	F03	Homo sapiens DKFZP564M082 protein	Yes
C12orf31								No
C13orf27	1388	93081	BC015148	561	11074	G11	Homo sapiens hypothetical protein BC015148	Yes
C14orf29								No
C15orf41	4866	84529	BC006254	552	11074	B10	Homo sapiens hypothetical protein HH114	Yes

Table 2-1 (Continued)

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
C1orf170	4901	84808	BC006300	177	11040	B06	Homo sapiens hypothetical protein MGC13275	Yes
C1QTNF6	429	114904	BC020551	837	11050	B09	Homo sapiens C1q and tumor necrosis factor related protein 6, transcript variant 1	Yes
C4orf42	859	92070	BC013775	276	11040	F01	Homo sapiens hypothetical protein MGC21675	Yes
C4orf42	56517	92070	BC120947	561	51017	A10	Homo sapiens chromosome 4 open reading frame 42, mRNA (cDNA clone MGC:149426 IMAGE:40115129), complete cds.	Yes
C5orf13	7787	9315	BC019068	207	11015	D04	Homo sapiens chromosome 5 open reading frame 13	No
CCDC86	6766	79080	BC001378	1083	11034	C03	Homo sapiens hypothetical protein MGC2574	No
CCL19	8385	6363	BC027968	297	11040	G01	Homo sapiens chemokine (C-C motif) ligand 19	Yes
CD99L2	8235	83692	BC025729	438	11027	C04	Homo sapiens CD99 antigen-like 2	Yes *
CD99L2	9718	83692	BC030536	789	11030	G04	Homo sapiens CD99 antigen-like 2	Yes *
CKS1B	4920	1163	BC007751, BC015629	240	11040	D06	Homo sapiens CDC28 protein kinase regulatory subunit 1B	Yes
CHMP4A	6411	29082	BC010893	669	11084	F04	Homo sapiens chromosome 14 open reading frame 123	Yes *
CHMP4A	53747	29082	BC113533, BC113535	798	51004	F06	Homo sapiens chromatin modifying protein 4A, mRNA (cDNA clone MGC:142095 IMAGE:8322587), complete cds.	Yes *
DRAM								No
ERLIN1	8101	10613	BC031791	1041	11056	F10	Homo sapiens chromosome 10 open reading frame 69	No
FXC1	6274	26515	BC011014	312	11082	D05	Homo sapiens fracture callus 1 homolog (rat)	Yes
GTF2A2	3117	2958	BC000287	330	11026	C05	Homo sapiens general transcription factor IIA, 2, 12kDa	No

Table 2-1 (Continued)

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
H2AFY	6390	9555	BC013331	1119	11086	F04	Homo sapiens H2A histone family, member Y	Yes
HMGB2	41	3148	BC000903	588	11045	A03	Homo sapiens high-mobility group box 2	Yes
HMGB2	6079	3148	BC001063	630	11029	D03	Homo sapiens high-mobility group box 2	Yes
HNMT	9025	3176	BC005907	156	11015	B03	Homo sapiens histamine N-methyltransferase	Yes
HNMT	13091	3176	BC020677	879	31020	A05	Homo sapiens histamine N-methyltransferase	Yes
IFITM2	3051	10581	BC009696	399	11026	H08	Homo sapiens interferon induced transmembrane protein 2 (1-8D)	No
IRF9	11201	10379	BC035716	1182	31006	G01	Homo sapiens interferon-stimulated transcription factor 3, gamma 48kDa	No
KHDC1	1648	80759	BC022080	492	11074	F01	Homo sapiens chromosome 6 open reading frame 148	Yes
LHX4	6869	89884	BC011759	1173	11059	H08	Homo sapiens LIM homeobox 4	No
LRRC28	8759	123355	BC019704	282	11040	F03	Homo sapiens hypothetical protein MGC24976	Yes
LRRC28	11470	123355	BC047306	1104	31009	D06	Homo sapiens leucine rich repeat containing 28	Yes
LSM6	241	11157	BC016026	243	11001	D02	Homo sapiens LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	Yes
MEI1	9586	150365	BC032248	717	11030	B11	Homo sapiens hypothetical protein MGC40042	Yes
MLF1IP	8728	79682	BC031520	531	11083	D10	Homo sapiens KSHV latent nuclear antigen interacting protein 1	Yes
MLF1IP	53246	79682	BC131556	1257	51029	E02	Homo sapiens MLF1 interacting protein, mRNA (cDNA clone MGC:148077 IMAGE:40108113), complete cds.	Yes

Table 2-1 (Continued)

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
MRPL42	2408	28977	BC040240	429	11042	F02	Homo sapiens mitochondrial ribosomal protein L42, transcript variant 2	No
NAF1	2691	92345	BC008207	1485	31045	H01	Homo sapiens hypothetical protein BC008207	Yes
NAPG	3684	8774	BC001889	939	11053	E01	Homo sapiens N-ethylmaleimide-sensitive factor attachment protein, gamma	Yes
NHP2L1	5568	4809	BC005358	387	11017	B08	Homo sapiens NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	No
NHP2L1	5987	4809	BC019282	387	11041	H02	Homo sapiens NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	No
NKIRAS2	4646	28511	BC007450, BC063498	576	11075	B02	Homo sapiens NFKB inhibitor interacting Ras-like 2, transcript variant 2	Yes
NUAK2	7863	81788	BC017306	1887	11070	G07	Homo sapiens likely ortholog of rat SNF1/AMP-activated protein kinase	Yes *
NXNL2	1150	158046	BC022521	408	11083	C01	Homo sapiens chromosome 9 open reading frame 121	Yes
PAFAH1B3	3838	5050	BC003016, BC007863	696	11046	H05	Homo sapiens platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa	Yes
PARP6	8620	56965	BC015176	330	11001	H10	Homo sapiens hypothetical protein from EUROMAGE 1977056	Yes
PARP6	9401	56965	BC026955	1092	11034	D06	Homo sapiens hypothetical protein from EUROMAGE 1977056	Yes
PEF1	4111	553115	BC002773	855	11050	F03	Homo sapiens PEF protein with a long N-terminal hydrophobic domain (peflin)	Yes
PEF1	6457	553115	BC012561	855	11006	E11	Homo sapiens PEF protein with a long N-terminal hydrophobic domain (peflin)	Yes

Table 2-1 (Continued)

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
PIGG	2759	54872	BC001249	1815	31045	H02	Homo sapiens hypothetical protein FLJ20265	Yes
PKN1	10163	5585	BC040061	2829	11080	F05	Homo sapiens protein kinase C-like 1	Yes
PTPLB	10939	201562	BC060839	765	31005	G03	Homo sapiens protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b	Yes
RNF181	4189	51255	BC002803	462	11073	F10	Homo sapiens hypothetical protein LOC51255	Yes
RPS4Y1	2371	6192	BC010286	792	11049	A05	Homo sapiens ribosomal protein S4, Y-linked 1	Yes
RPS4Y1	55775	6192	BC100905	792	51018	A09	Homo sapiens ribosomal protein S4, Y-linked 1, mRNA (cDNA clone MGC:119100 IMAGE:40003602), complete cds.	Yes
RPS6	4678	6194	BC000524	750	11030	D11	Homo sapiens ribosomal protein S6	Yes
RPS6	6020	6194	BC013296	750	11030	D07	Homo sapiens ribosomal protein S6	Yes
RPS6	13163	6194	BC027620	750	31019	F10	Homo sapiens ribosomal protein S6	Yes
SELI								Yes
SF3B14	788	51639	BC015463	378	11026	F11	Homo sapiens pre-mRNA branch site protein p14	No
SLC25A17	5682	10478	BC005957, BC012998	924	11053	A07	Homo sapiens solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kDa), member 17	Yes
SMCP	1446	4184	BC014593, BC016744	351	11041	D08	Homo sapiens mitochondrial capsule selenoprotein	No
SNAI1	2620	6615	BC012910	795	11006	A08	Homo sapiens snail homolog 1 (Drosophila)	Yes
SPINK2	1251	6691	BC022514	255	11081	A12	Homo sapiens serine protease inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	Yes
STEAP1	6967	26872	BC011802	1020	31043	D04	Homo sapiens six transmembrane epithelial antigen of the prostate	Yes

Table 2-1 (Continued)

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
STK40	5141	83931	BC007835	1308	11062	G06	Homo sapiens Ser/Thr-like kinase	Yes
STK40	5312	83931	BC008344	702	11020	B12	Homo sapiens Ser/Thr-like kinase	Yes
TAC1	1164	6863	BC018047	390	11026	H02	Homo sapiens tachykinin precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma), transcript variant beta	No
TCEB1	839	6921	BC013809	339	11041	B12	Homo sapiens transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	Yes
TEX13A	11118	56157	BC042547	1230	31006	G02	Homo sapiens testis expressed sequence 13A	Yes
TEX261	7901	113419	BC020251	450	11017	G10	Homo sapiens testis expressed gene 261	Yes
TEX261	56894	113419	BC128461	591	51031	B08	Homo sapiens testis expressed 261, mRNA (cDNA clone MGC:156306 IMAGE:40126644), complete cds.	Yes
TMSB4Y	1130	9087	BC022482	135	11081	A03	Homo sapiens thymosin, beta 4, Y-linked	Yes
TNFRSF21	348	27242	BC010241	1419	11012	D01	Homo sapiens tumor necrosis factor receptor superfamily, member 21	Yes
TNFRSF21	484	27242	BC017730	1968	11013	H08	Homo sapiens tumor necrosis factor receptor superfamily, member 21	Yes
TNFRSF21	8621	27242	BC005192, BC015466	375	11017	A03	Homo sapiens tumor necrosis factor receptor superfamily, member 21	Yes
TRADD	4340	8717	BC004491	939	31044	H02	Homo sapiens TNFRSF1A-associated via death domain, transcript variant 1	Yes
TRPV6	9356	55503	BC034814	321	11001	H05	Homo sapiens transient receptor potential cation channel, subfamily V, member 6	Yes
TSPAN14	4436	81619	BC002920	762	11048	A10	Homo sapiens tetraspanin similar to TM4SF9	Yes

Table 2-1 (Continued)

Symbol	Internal ID	ENTREZ Gene ID	BC NUMBER	ORF Length	Resource Plate	Resource Position	Description	Verified using secondary assay
TUBA1B	229	10376	BC000696, BC001128, BC006379, BC006481, BC008659, BC009314, BC009509, BC009513, BC010494, BC011572, BC015883	1356	11011	F12	Homo sapiens tubulin, alpha, ubiquitous	Yes
TUBA1B	1072	10376	BC017004, BC030820	1356	11011	F11	Homo sapiens tubulin, alpha, ubiquitous	Yes
TUBA1B	6115	10376	BC009512	1356	11011	G01	Homo sapiens tubulin, alpha, ubiquitous	Yes
TUBA1B	7910	10376	BC021564	1008	11033	B11	Homo sapiens tubulin, alpha, ubiquitous	Yes
TUBA1B	14965	10376	BC071904	1356	31034	G09	Homo sapiens tubulin, alpha, ubiquitous, mRNA (cDNA clone MGC:88583 IMAGE:6089096), complete cds.	Yes
UPK3B	4272	80761	BC004304	963	11054	C04	Homo sapiens uroplakin 3B, transcript variant 1	No
VGLL4	5837	9686	BC001514	873	11051	A06	Homo sapiens Vestigial-like 4	Yes *
WDR42A								Yes
WRB	637	7485	BC012415	525	11044	A03	Homo sapiens tryptophan rich basic protein	Yes
ZFYVE21	5764	79038	BC005999	705	11020	C08	Homo sapiens zinc finger, FYVE domain containing 21	Yes
ZFYVE21	6090	79038	BC001130	705	11030	B03	Homo sapiens zinc finger, FYVE domain containing 21	Yes
ZNF271	1025	10778	BC017710	1272	11062	A07	Homo sapiens zinc finger protein 271	Yes

2.3 Secondary screen

2.3.1. Introduction

Our primary screen using a human ORFeome library allowed us to identify 75 preliminary candidates. Individual verification of these candidates was necessary for several reasons: a) PCR analysis on isolated colonies revealed that the majority of the isolated colonies had more than one insertion. This observation can be explained by the fact that the primary screen was performed in a pooled fashion at an MOI of approximately 3.5. Additionally, ORFeome-transduced cells were passaged twice, raising the possibility of having colonies that are a mixture of different populations of cells with different insertions. b) In order to find the most robust subset of candidates to concentrate on for further studies and validation, we were interested in those that had a dominant effect independent of other factors.

2.3.2. Experimental Design

A. Establishment of permissive conditions

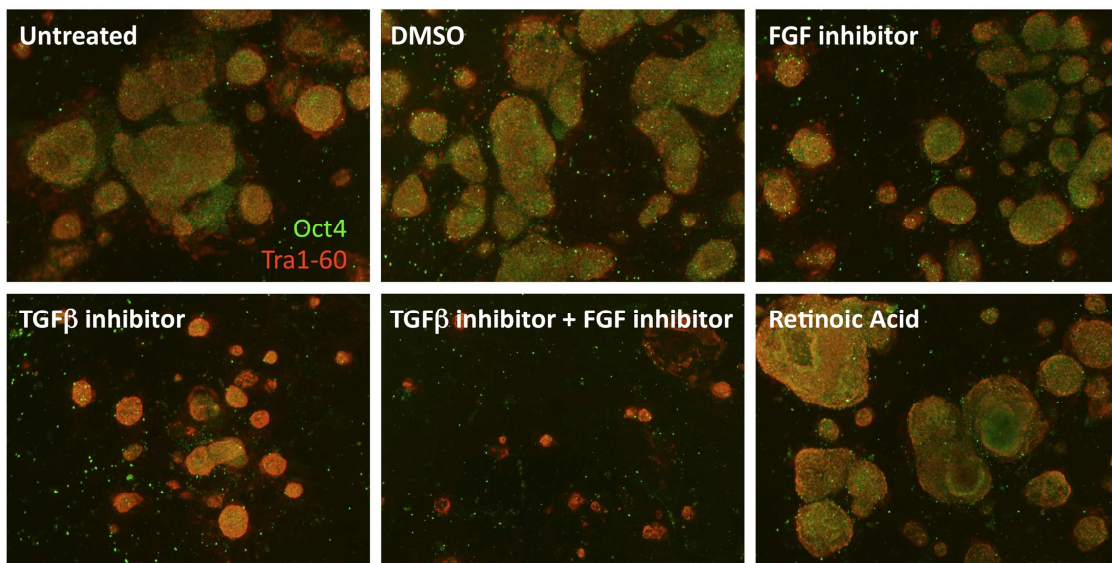
We aimed to find conditions of increased stringency that would allow us to verify our preliminary candidates in a fast and efficient manner. To this end, we sought to identify small molecule inhibitors for pathways known to be important for hES cell pluripotency that, when used in combination, with SB-431532 led to a more rapid loss of hES cell morphology.

TGF β and FGF signaling have been shown to be necessary for hES cell pluripotency [5, 6, 16-18]. Additionally, several studies have uncovered evidence for a synergistic

activity of these pathways for maintaining the pluripotent state [7-9]. Therefore, we tested SU-5402, an inhibitor for the kinase activity of FGFR1 [19], could rapidly induce differentiation, either alone or in combination with SB-431542 for 5 and 7 days. Retinoic acid was also tested given its differentiation-inducing abilities [20, 21].

We found that SB-431542 and SU-5402 alone caused little differentiation after 5 or 7 days as assessed by colony morphology, Oct4, and Tra 1-60 expression. Retinoic acid also had only a very mild effect at these timepoints. However, the combination of SB-431542 and SU-5402 caused rapid and robust differentiation, assessed by loss of colony morphology and widespread downregulation of Oct4. This differentiation was evident by 5 days, making this our timepoint of choice (**Figure 2-5**).

5 days of treatment



7 days of treatment

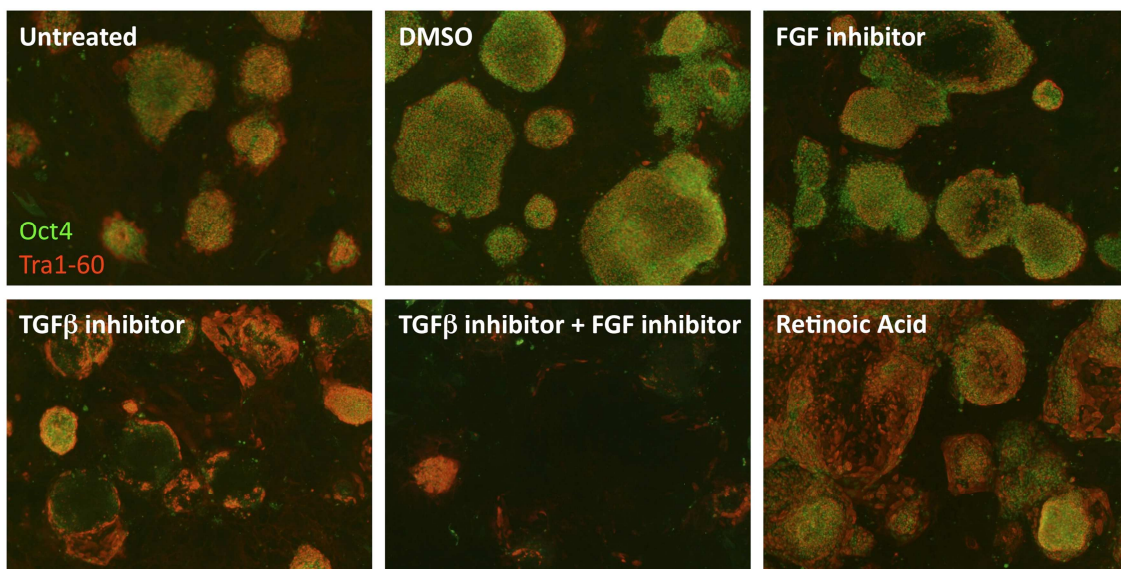


Figure 2-5. Optimization of conditions for secondary verification assay. A TGFβ inhibitor (SB-431542), an FGF inhibitor (SU-5402), and Retinoic Acid were tested for their ability to cause differentiation in 5 or 7 days. Differentiation was assessed by evaluating colony morphology, Oct4, and Tra 1-60 expression. At both timepoints, FGF_i and Retinoic Acid had a mild effect. Treatment with TGFβ_i caused some differentiation, but a considerable number of undifferentiated cells were still present at both timepoints. The combination of TGFβ_i and FGF_i caused robust loss of pluripotency marker expression and colony morphology by 5 days.

We also verified the double inhibitor treatment [10 μ M TGF β i (SB-431542) and 20 μ M FGF β i (SU-5402) –referred to throughout this thesis as TGF β i+FGF β i for TGF β inhibitor and FGF inhibitor] using our Nanog and GFP controls. In the presence of Nanog, cells maintained Oct4 expression and colonies retained normal morphology. As expected, the GFP control exhibited differentiation much like its wildtype counterpart, with only scattered pockets of Oct4-positive cells remaining (**Figure 2-6**).

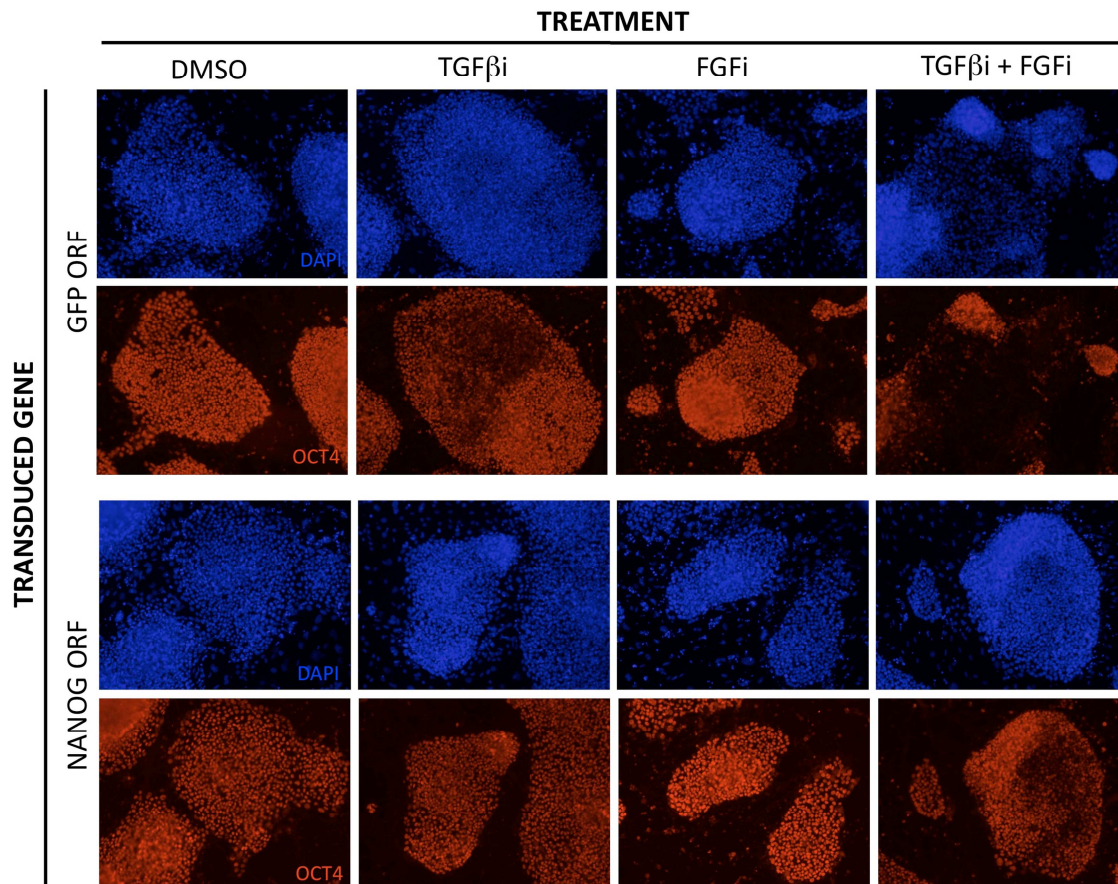


Figure 2-6. Verification of secondary screening conditions using overexpression controls. Combined inhibition of TGFβ and FGF causes Oct4 downregulation and loss of colony morphology (as shown by DAPI panel) in cells overexpressing GFP but not in cells overexpressing Nanog. GFP control cells show little differentiation after 5 days of treatment with TGFβi or FGFi alone.

B. Candidate verification

Optimization of the conditions for secondary verification of our preliminary candidates allowed for a rapid and stringent verification of those that could overcome inhibition of two pathways necessary for pluripotency maintenance. To verify the candidates, lentiviruses were produced for each individual candidate and used to transduce cells at an MOI of 1-2. We then selected transduced cells using 2 µg/ml of puromycin. Following selection, cells were treated with TGFβi+FGFi for 5 days with media changes every second day. After this treatment, colony morphology, Oct4, and Tra 1-60 expression were assessed (**Figure 2-7**).

We verified 56 out of the 75 preliminary candidates using 5 rounds of verification in duplicates (**Figure 2-8**).

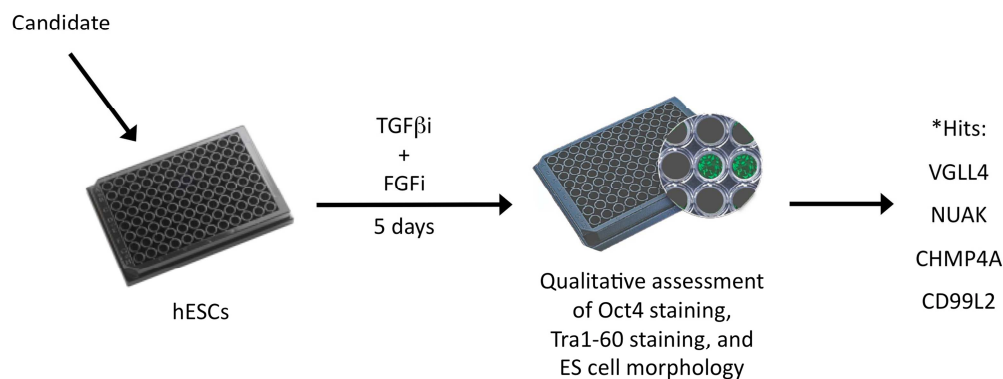


Figure 2-7. Identification of four positive hits using a secondary verification assay. Preliminary candidates were verified using a more stringent test for maintenance of the hES cell state. The verification assay consisted of 5 days of treatment with 10µM TGFβi in addition to 20µM FGF inhibitor (FGFi). DMSO was used as a vehicle control. After this treatment, qualitative analysis of colony morphology and expression of ES cell markers Oct4 and Tra 1-60 was assessed by immunostaining. Hits indicated with an asterisk here, and in Table 1-1, were those that were identified after the secondary verification assay.

2.3.3. Results

We identified four positive hits from our secondary verification assay.

A. *Vestigial-like 4 (Vgll4)*

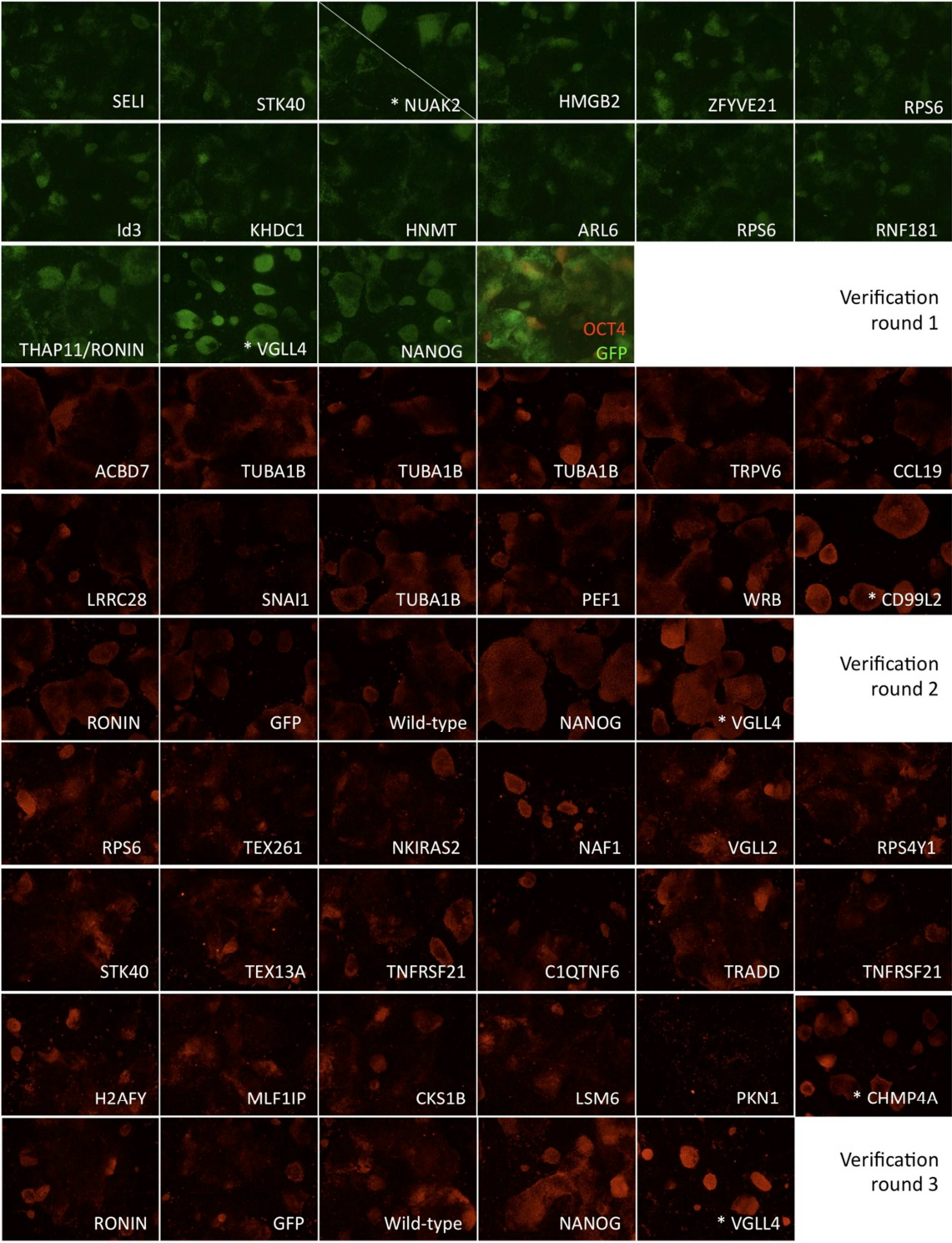
Vgll4 is a co-transcriptional regulator of Transcription Enhancer Factors (TEF) transcription factors. The only described function for Vgll4 in mammals to date is a role in regulating transcription downstream of α -1 adrenergic signaling in cardiomyocytes [22]. Vgll4 has no previous roles described in hES cells. For a complete description of Vgll4, please refer to Chapter 3.

I chose to concentrate on Vgll4 for my characterization for two reasons. First, it was the only candidate that was identified multiple times in the primary screen, an event of very low probability that underscores a powerful effect. Second, Vgll4 is the only transcriptional regulator out of our positive hits. I was interested in an effect closer to the transcriptional network of hES cells.

Vgll4 consistently maintained colony morphology and high levels of Oct4 after treatment with the combination of inhibitors (TF) (**Figure 2-9**). Additionally, Vgll4-overexpressing cells maintained expression of a panel of pluripotency markers after treatment with the inhibitor combination (**Figure 2-10**).

Figure 2-8. Individual verification of preliminary candidates reveals positive hits for pluripotency maintenance. Three independent rounds of candidate verification are shown. Images show Oct4 staining in the samples treated with the combination of inhibitors for five days. We also included other pluripotency-related genes that were not originally in our list of preliminary candidates. We performed a qualitative evaluation of colony morphology and proportion of Oct4 expression to identify positive hits. Vgll4 consistently retains Oct4 expression and colony morphology. An asterisk marks the genes identified as hits. Chmp4a was part of verification round 4.

Figure 2-8 (Continued)



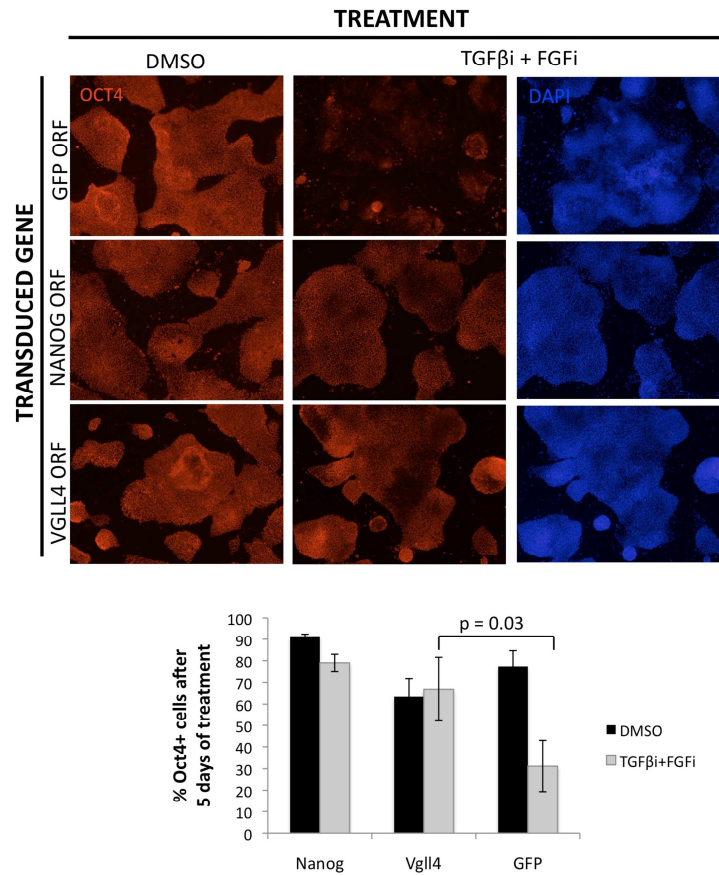


Figure 2-9. Vgll4 is a co-transcriptional regulator that can maintain hES cell identity after treatment with inhibitors. Cells transduced with Vgll4 or control genes were treated with vehicle control or with the inhibitor mix for 5 days and stained for Oct4 at the end of the treatment. Quantification of Oct4⁺ cells after treatment using Cellomics image acquisition and software (DMSO: vehicle control).

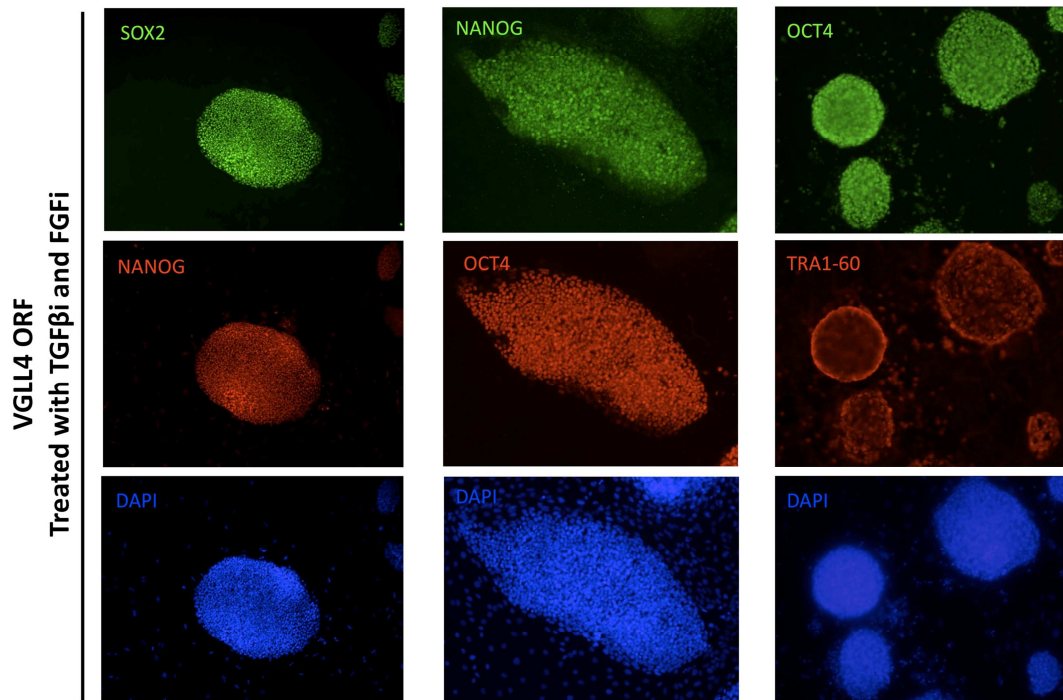


Figure 2-10. Vgll4 maintains expression of pluripotency markers after treatment with TGFβi and FGFi. Cells overexpressing Vgll4 and treated with the combination of inhibitors for 5 days are able to maintain expression of a panel of pluripotency markers including transcription factors Oct4, Sox2, and Nanog as well as surface markers like Tra 1-60.

B. CD99 Antigen-like 2 (CD99L2)

CD99L2 is a paralog of the surface protein CD99. In contrast to CD99, CD99L2 is strongly conserved among human, rat, mouse, and zebrafish [23]. The only described role to date for CD99L2 is that, much like CD99, it is necessary for migration of leukocytes across endothelial cells during the inflammatory response [24]. Additionally, CD99L2 is sufficient to mediate cation-dependent cell adhesion. This suggests a role in mediating cell adhesion in certain cell populations.

C. Charged multivesicular body protein 4A (CHMP4A)

CHMP4A belongs to the chromatin modifying protein/charged multivesicular protein family. These proteins form part of the endosomal sorting complex required for transport-III (ESCRT-III), which degrades surface receptor proteins and forms multivesicular bodies [25]. CHMP4A has been shown to have a role in membrane curvature and budding [26].

D. NIAK family, SNF1-like kinase 2 (NIAK2)

NIAK2 is a member of the SNF1/AMP family of serine/threonine kinases. This family of kinases is involved in sensing the energy levels in the cell and coupling that information with growth and proliferation [27]. Therefore, when deregulated, this gene can contribute to tumor progression. This family of kinases is known to be regulated by tumor suppressor LKB1 and by the death receptor through NF- κ B [28]. Additionally, NIAK2 has been shown to have a role in regulating actin stress fibers, especially in exponentially-growing cells [29].

2.4 Discussion

We were interested in implementing a broad approach to discover genes involved in the maintenance of the pluripotent state in hES cells. To date, some screens have explored finding pluripotency genes in hES cells [30]. However, these have mostly focused on a loss-of-function approach. We designed a screen based on the inhibition of TGF β signaling to identify positive hits in a genetic gain-of-function screen. Our positive and negative controls were validated in these conditions. We identified 75 preliminary candidates that were subsequently validated in a secondary assay of higher stringency. Four hits were identified and we decided to focus on Vgll4 given its role in transcription regulation. Our screen provides a proof-of-principle that gain-of-function screens using lentiviruses are an efficient approach to gene discovery and characterization in hES cells.

Our list of primary candidates represents approximately 1% of genes contained in our initial library. Vgll4, the candidate that was chosen for further characterization, was identified three independent times in the primary screen. This is a very low probability event (1×10^{-6}), suggesting a robust effect for Vgll4. In addition, Vgll4 was the only gene out of the 75 preliminary candidates that was identified multiple times. In the future, to know if one is operating close to saturation, the positive control (Nanog, in this case) could be included at known low quantities in the screening pool.

Our calculations predict that the library should be represented about 1000 times, however, it is possible that we are still operating below saturation. This means that many more ORFs contained in our screened pool could have been missed as primary

candidates. While operating below saturation possibly allowed us to find the genes with the most robust effect, it would be interesting to find more primary hits. Additionally, since the time we carried out these experiments, the Center for Cancer Systems Biology Human ORFeome Consortium (Dana Farber Cancer Institute, Harvard University) has derived version 7.1 of the human ORFeome, containing close to 20, 000 ORFs representing over 15, 000 genes. If a screen were to be repeated with those larger pools using these same optimized conditions, many more genes with a potentially important role in pluripotency could be identified.

For many genes involved in the regulation of pluripotency, expression at specific levels is of utmost importance. Nanog is one of the few genes that can be overexpressed for long periods of time without altering the pluripotent potential of hESCs [1, 11]. Timing is an important factor to consider when optimizing screening conditions. Our initial screening conditions were stringent in the sense that if a gene causes differentiation when overexpressed, but still has an important role in pluripotency, it would likely not be recovered using our conditions. For instance, levels of Oct4 and Sox2 need to be carefully regulated for human and mouse ES cells to remain pluripotent [12, 13, 31]. Therefore, one could ponder that perhaps our longer primary screening conditions are also selecting for genes involved with survival and overcoming stress under differentiation cues rather than maintaining a fine balance of gene products in the pluripotent state. Interestingly, some of our primary candidates seem to have a role in regulating apoptosis and the stress response. High levels of cell death are consistently observed in the first few days of hES cell differentiation. It is therefore

possible that by inducing differentiation, we are not only creating permissive conditions for certain pluripotency or self-renewal genes, but also for genes involved in survival.

2.5 Materials and Methods used in this chapter

2.5.1. Culture of *hES* cells

HUES6 were plated on irradiated murine embryonic fibroblasts (MEFs) and grown in media containing KO-DMEM (Invitrogen), 10% Knockout Serum Replacement (KOSR, GIBCO), 10% human plasma fraction (Talecris), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (GIBCO), .055 mM β -Mercaptoethanol (GIBCO), and 10 ng/ml bFGF (Invitrogen). Cultures were passaged using 0.05% Trypsin-EDTA (GIBCO) at a ratio of 1:6-1:10 every 5-7 days.

Where indicated, SB-431542 (Sigma Aldrich) was used at a concentration of 10 μ M, SU-5402 (Tocris) at 20 μ M. Both of these were resuspended in DMSO. DMSO vehicle controls were made with the equivalent volume of DMSO of the two chemicals combined. Retinoic Acid was used at a final concentration of 10 μ M.

MEFs were plated on plates coated with 0.1% gelatin. MEF media contained 1X DMEM (Mediatech), 10% FBS, 2mM L-glutamine (Invitrogen), and 0.1mM non-essential amino acids (GIBCO).

2.5.2. *Lentivirus* production

293T/17 cells were grown in DMEM (Mediatech), 10%FBS, 2 mM L-glutamine (Invitrogen), and 0.1 mM non-essential amino acids (GIBCO). A day before transfection, cells were plated at a concentration of ~ 85000 cells/cm² (or 8.0×10^5 per well of a 6-well dish). 20 hours later a media change was done on the cells. 2 hours after adding fresh media, the cells were transfected with a total of 2 μ g DNA per well of a 6-well dish.

A third-generation packaging system consisting of tat, rev, gag/pol and VSVG was mixed in a ratio of 5:1:1:1:2 (DNA:tat:rev:gag/pol:VSVG). As per manufacturer's instructions, 5 µl of TRANS-IT 293 (MirusBio) and 167 µl Optimem (GIBCO) were mixed thoroughly and incubated at room temperature for 5-10 minutes. Following this incubation, 172 µl of Mirus/Optimem mix was added to the DNA/packaging plasmid mix with very gentle mixing and incubated for 20 minutes at room temperature. After this incubation, the mix was added dropwise onto the cells and mixed gently by moving the plate back and forth. A media change was performed 24 hours later. Virus was harvested 24 hours later, filtered through a low-protein binding 0.45 µm filter, and added to cells or flash frozen in liquid nitrogen and stored at -80°C until use.

2.5.3. hES cell transduction

Cells were MEF-depleted for 30-45 min on gelatin-coated plates. Cells in suspension were collected, counted, and mixed with harvested lentivirus at an MOI of 2. Cells were incubated with the virus in a low-attachment dish for 2-3 hours at 37°C and 5% CO₂ with occasional rocking. After this time, the cells were pelleted by centrifugation at 1000 rpm for 5 minutes at room temperature. The cells were then plated onto Puromycin-resistant MEFs. 48 hours later, transduced cells were selected with 2 µg/ml of puromycin for 2 days, generating a population of >98% transduced cells.

2.5.4. Flow cytometry analysis

Cells treated with DMSO or 10 μ M SB-431542 were harvested using 0.5% trypsin. Cells were washed once in PBS and then incubated in 100 μ l of a 1:100 dilution of mouse IgM anti-Tra 1-60 (Millipore) or rat IgM anti-SSEA-3 (Santa Cruz Biotechnology) in FACS buffer (2% Hyclone Fetal Calf Serum in PBS). Cells were incubated in primary antibody for 15-30 minutes on ice. After this incubation, cells were washed with PBS and stained with secondary antibodies conjugated to APC (Jackson ImmunoResearch) at a dilution of 1:300 for 15 minutes on ice. Cells were washed with PBS and resuspended in FACS buffer. Cells were filtered immediately before analysis through a 35 μ m filter. An LSRII was used for analysis.

2.5.5. Immunofluorescent staining

Cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Two washes were performed after fixation. Cells were blocked for 1 hour at room temperature or overnight at 4°C in 5% donkey serum in 0.1% PBST (0.1% TritonX-100 in PBS). Cells were incubated with primary antibodies in block for 2 hours at room temperature or overnight at 4°C. Primary antibodies used are: goat anti-Oct-4, 1:200 (Santa Cruz biotechnology); rabbit anti-Nanog, 1:50 (R&D); mouse anti-Sox2 1:200 (Cell Signaling Technologies), mouse IgM anti-Tra 1-60 (Millipore). Primary antibody was washed 2-3 times with 0.1% PBST. Next, cells were incubated for 1 hour at room temperature with secondary antibodies raised in donkey and conjugated to Alexa fluorescent probes. All secondary antibodies were used at a 1:300 dilution. Cell nuclei were stained using DAPI at 1 μ g/ml.

2.6 Note regarding author contributions

Dr. René Maehr subcloned the hORFeome library into the Gateway-optimized pHAGE vector. He also contributed to the design of the primary and verification screens.

References

- [1] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, and A. Smith, "Functional Expression Cloning of Nanog, a Pluripotency Sustaining Factor in Embryonic Stem Cells," *Cell*, vol. 113, pp. 643-655, 2003.
- [2] G. J. Murphy, G. Mostoslavsky, D. N. Kotton, and R. C. Mulligan, "Exogenous control of mammalian gene expression via modulation of translational termination," *Nat Med*, vol. 12, pp. 1093-1099, 2006.
- [3] A. A. Wilson, G. J. Murphy, H. Hamakawa, L. W. Kwok, S. Srinivasan, A.-H. Hovav, R. C. Mulligan, S. Amar, B. Suki, and D. N. Kotton, "Amelioration of emphysema in mice through lentiviral transduction of long-lived pulmonary alveolar macrophages," *The Journal of Clinical Investigation*, vol. 120, pp. 379-389, 2010.
- [4] X. Zeng, J. Chen, J. F. Sanchez, M. Coggiano, O. Dillon-Carter, J. Petersen, and W. J. Freed, "Stable Expression of hrGFP by Mouse Embryonic Stem Cells: Promoter Activity in the Undifferentiated State and During Dopaminergic Neural Differentiation," *Stem Cells*, vol. 21, pp. 647-653, 2003.
- [5] G. M. Beattie, A. D. Lopez, N. Bucay, A. Hinton, M. T. Firpo, C. C. King, and A. Hayek, "Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers," *Stem Cells*, vol. 23, pp. 489-495, April 1, 2005 2005.
- [6] D. James, A. J. Levine, D. Besser, and A. Hemmati-Brivanlou, "TGF β /activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells," *Development*, vol. 132, pp. 1273-1282, March 15, 2005 2005.
- [7] L. Vallier, M. Alexander, and R. A. Pedersen, "Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells," *J Cell Sci*, vol. 118, pp. 4495-4509, October 1, 2005 2005.
- [8] L. Xiao, X. Yuan, and S. J. Sharkis, "Activin A Maintains Self-Renewal and Regulates Fibroblast Growth Factor, Wnt, and Bone Morphogenic Protein Pathways in Human Embryonic Stem Cells," *Stem Cells*, vol. 24, pp. 1476-1486, June 1, 2006 2006.
- [9] R. H. Xu, T. L. Sampsel-Barron, F. Gu, S. Root, R. M. Peck, G. Pan, J. Yu, J. Antosiewicz-Bourget, S. Tian, R. Stewart, and J. A. Thomson, "NANOG is a direct target of TGF β /activin-mediated SMAD signaling in human ESCs," *Cell Stem Cell*, vol. 3, pp. 196-206, Aug 7 2008.

- [10] G. J. Inman, F. J. Nicolás, J. F. Callahan, J. D. Harling, L. M. Gaster, A. D. Reith, N. J. Laping, and C. S. Hill, "SB-431542 Is a Potent and Specific Inhibitor of Transforming Growth Factor-beta Superfamily Type I Activin Receptor-Like Kinase (ALK) Receptors ALK4, ALK5, and ALK7," *Molecular Pharmacology*, vol. 62, pp. 65-74, July 1, 2002 2002.
- [11] H. Darr, Y. Mayshar, and N. Benvenisty, "Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features," *Development*, vol. 133, pp. 1193-1201, March 15, 2006 2006.
- [12] H. Niwa, J.-i. Miyazaki, and A. G. Smith, "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells," *Nat Genet*, vol. 24, pp. 372-376, 2000.
- [13] J. L. Kopp, B. D. Ormsbee, M. Desler, and A. Rizzino, "Small Increases in the Level of Sox2 Trigger the Differentiation of Mouse Embryonic Stem Cells," *Stem Cells*, vol. 26, pp. 903-911, 2008.
- [14] B. Boer, J. Kopp, S. Mallanna, M. Desler, H. Chakravarthy, P. J. Wilder, C. Bernadt, and A. Rizzino, "Elevating the levels of Sox2 in embryonal carcinoma cells and embryonic stem cells inhibits the expression of Sox2:Oct-3/4 target genes," *Nucleic Acids Research*, vol. 35, pp. 1773-1786, March 1, 2007 2007.
- [15] J. F. Rual, T. Hirozane-Kishikawa, T. Hao, N. Bertin, S. Li, A. Dricot, N. Li, J. Rosenberg, P. Lamesch, P. O. Vidalain, T. R. Clingingsmith, J. L. Hartley, D. Esposito, D. Cheo, T. Moore, B. Simmons, R. Sequerra, S. Bosak, L. Doucette-Stamm, C. Le Peuch, J. Vandenhaute, M. E. Cusick, J. S. Albala, D. E. Hill, and M. Vidal, "Human ORFeome version 1.1: a platform for reverse proteomics," *Genome Res*, vol. 14, pp. 2128-35, Oct 2004.
- [16] P. Dvorak, D. Dvorakova, S. Koskova, M. Vodinska, M. Najvirtova, D. Krekac, and A. Hampl, "Expression and Potential Role of Fibroblast Growth Factor 2 and Its Receptors in Human Embryonic Stem Cells," *Stem Cells*, vol. 23, pp. 1200-1211, September 1, 2005 2005.
- [17] C. Xu, E. Rosler, J. Jiang, J. S. Lebkowski, J. D. Gold, C. O'Sullivan, K. Delavan-Boorsma, M. Mok, A. Bronstein, and M. K. Carpenter, "Basic Fibroblast Growth Factor Supports Undifferentiated Human Embryonic Stem Cell Growth Without Conditioned Medium," *Stem Cells*, vol. 23, pp. 315-323, March 1, 2005 2005.
- [18] R.-H. Xu, R. M. Peck, D. S. Li, X. Feng, T. Ludwig, and J. A. Thomson, "Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells," *Nat Meth*, vol. 2, pp. 185-190, 2005.
- [19] M. Mohammadi, G. McMahon, L. Sun, C. Tang, P. Hirth, B. K. Yeh, S. R. Hubbard, and J. Schlessinger, "Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors," *Science*, vol. 276, pp. 955-60, May 9 1997.
- [20] A. L. Goulburn, D. Alden, R. P. Davis, S. J. Micallef, E. S. Ng, Q. C. Yu, S. M. Lim, C.-L. Soh, D. A. Elliott, T. Hatzistavrou, J. Bourke, B. Watmuff, R. J. Lang, J. M. Haynes, C.

- W. Pouton, A. Giudice, A. O. Trounson, S. A. Anderson, E. G. Stanley, and A. G. Elefanty, "A Targeted NKX2.1 Human Embryonic Stem Cell Reporter Line Enables Identification of Human Basal Forebrain Derivatives," *Stem Cells*, vol. 29, pp. 462-473.
- [21] T. Ryan, J. Liu, A. Chu, L. Wang, A. Blais, and I. Skerjanc, "Retinoic Acid Enhances Skeletal Myogenesis in Human Embryonic Stem Cells by Expanding the Premyogenic Progenitor Population," *Stem Cell Reviews and Reports*, pp. 1-12, 2011.
- [22] H.-H. M. Chen, Steven J.; Stewart, Alexandre F.R., "Vgl-4, a Novel Member of the Vestigial-like Family of Transcription Cofactors, Regulates (alpha one)-Adrenergic Activation of Gene Expression in Cardiac Myocytes," *Journal of Biological Chemistry*, vol. 279, pp. 30800-30806, July 16, 2004 2004.
- [23] Y. H. Suh, Y. K. Shin, M.-C. Kook, K. I. Oh, W. S. Park, S. H. Kim, I.-S. Lee, H. J. Park, T.-L. Huh, and S. H. Park, "Cloning, genomic organization, alternative transcripts and expression analysis of CD99L2, a novel paralog of human CD99, and identification of evolutionary conserved motifs," *Gene*, vol. 307, pp. 63-76, 2003.
- [24] A. R. Schenkel, E. M. Dufour, T. W. Chew, E. Sorg, and W. A. Muller, "The Murine CD99-Related Molecule CD99-Like 2 (CD99L2) Is an Adhesion Molecule Involved in the Inflammatory Response," *Cell Communication and Adhesion*, vol. 14, pp. 227-237, 2007.
- [25] J. W. Peck, E. T. Bowden, and P. D. Burbelo, "Structure and function of human Vps20 and Snf7 proteins," *Biochem J*, vol. 377, pp. 693-700, Feb 1 2004.
- [26] P. I. Hanson, R. Roth, Y. Lin, and J. E. Heuser, "Plasma membrane deformation by circular arrays of ESCRT-III protein filaments," *J Cell Biol*, vol. 180, pp. 389-402, Jan 28 2008.
- [27] T. Namiki, A. Tanemura, J. C. Valencia, S. G. Coelho, T. Passeron, M. Kawaguchi, W. D. Vieira, M. Ishikawa, W. Nishijima, T. Izumo, Y. Kaneko, I. Katayama, Y. Yamaguchi, L. Yin, E. C. Polley, H. Liu, Y. Kawakami, Y. Eishi, E. Takahashi, H. Yokozeki, and V. J. Hearing, "AMP kinase-related kinase NUA2 affects tumor growth, migration, and clinical outcome of human melanoma," *Proceedings of the National Academy of Sciences*, vol. 108, pp. 6597-6602, April 19, 2011.
- [28] P. Legembre, R. Schickel, B. C. Barnhart, and M. E. Peter, "Identification of SNF1/AMP Kinase-related Kinase as an NF-kB-regulated Anti-apoptotic Kinase Involved in CD95-induced Motility and Invasiveness," *Journal of Biological Chemistry*, vol. 279, pp. 46742-46747, November 5, 2004 2004.
- [29] T. Vallenius, K. Vaahtomeri, B. Kovac, A.-M. Osiceanu, M. Viljanen, and T. P. M. V. S. K., "An association between NUA2 and MRIP reveals a novel mechanism for regulation of actin stress fibers," *Journal of Cell Science*, vol. 124, pp. 384-393, February 1, 2011.
- [30] N. Y. Chia, Y. S. Chan, B. Feng, X. Lu, Y. L. Orlov, D. Moreau, P. Kumar, L. Yang, J. Jiang, M. S. Lau, M. Huss, B. S. Soh, P. Kraus, P. Li, T. Lufkin, B. Lim, N. D. Clarke, F.

- Bard, and H. H. Ng, "A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity," *Nature*, vol. 468, pp. 316-20, Nov 11 2010.
- [31] R. T. Rodriguez, J. M. Velkey, C. Lutzko, R. Seerke, D. B. Kohn, K. S. O'Shea, and M. T. Firpo, "Manipulation of OCT4 Levels in Human Embryonic Stem Cells Results in Induction of Differential Cell Types," *Experimental Biology and Medicine*, vol. 232, pp. 1368-1380, November 1, 2007 2007.

Chapter 3

Vgll4 is a novel regulator of survival in human embryonic stem cells

3.1 Introduction

3.1.1. Vestigial-like proteins are transcriptional co-regulators that mediate the activity of Transcriptional Enhancer Factors

Vgll4 is a member of the Vestigial-like protein family, which contains four genes (Vgll1-4) in mice and humans. Vestigial-like proteins are transcriptional co-regulators that mediate the activity and determine the tissue specific gene control of Transcriptional Enhancer Factors (TEFs), also known as TEA domain-containing factors (TEADs) [1-3]. TEF/TEAD family members contain a TEA domain that allows them to bind DNA [4]. They regulate transcription of genes in multiple tissues, and it is thought that the co-transcriptional regulators they interact with confer their specificity [5]. Vgll

proteins contain a tondu motif (TDU), a conserved domain through which they interact with TEF factors.

The Vgll family of proteins is named for the founding member of the gene family, the Vestigial (Vg) gene in *D. melanogaster*. In *Drosophila*, loss of Vg prevents wing development and interferes with cell proliferation in the precursor cells of the wing imaginal disc [6, 7]. This suggests that mammalian orthologs of Vg might also have a role in the maintenance of multipotent cell populations.

Vgll4 is the only member of the Vgll family with two TDU motifs instead of one. The functional relevance of this double motif is not well understood, but some studies have suggested a role in bridging two transcription factors, for instance TEF-1 and MEF-2 [1]. The function of Vgll4 remains largely unstudied apart from a described role in transcriptional regulation in developing cardiomyocytes. In this study, Vgll4 was shown to interact with MEF-2 and TEF-1 in vitro. Additionally, the authors suggest nuclear export as a means for regulating Vgll4 function in cardiac myocytes [1].

3.1.2. The Rho/Rock pathway prevents the death of hES cells upon dissociation

One of the differences between mouse and human ES cells is their sensitivity to dissociation. While mouse ES cells flourish when cultured at clonal densities, hES cells die as single cells [8-10]. This sensitivity to dissociation is a major roadblock for many applications of hES cells that require growth from single cells. In hES cells, E-cadherin dependent cell-to-cell contacts inhibit the function of Abr, an upstream regulator of Rac and Rho. Abr has Rho-GEF and Rac-GAP domains. When E-cadherin-mediated cell-to-

cell contacts are disrupted, Abr generates a Rho-high/Rac-low state, which leads to activation of Rock through Rho. Rock activation results in myosin hyperactivation, which is manifested as membrane blebbing and results in caspase-mediated apoptosis [8, 11, 12]. For a graphical summary of this pathway, please refer to Chapter 1, Figure 1-2).

3.2 Characterization of Vgll4 expression

3.2.1. Expression of Vgll4 in hES cells

Vgll4 was identified in our screen as a gene that was able to maintain pluripotency marker expression and hES cell colony morphology despite inhibition of the TGF β /Activin/Nodal and FGF pathways, which have shown to be important for pluripotency maintenance. We first wanted to assess whether Vgll4 is normally expressed in hES cells and if it is specific to the pluripotent state. To this end, the relative expression of Vgll4 was quantified in hES cells by using real-time PCR analysis and immunofluorescence. Immunostaining analysis with a custom-made antibody (please refer to Methods section for details) revealed that Vgll4 localizes to the nucleus of Oct4 positive cells. Additionally, to assess expression in early differentiated populations, hES cells were differentiated in a three-dimensional system to form embryoid bodies. This analysis revealed expression of Vgll4 in hES cells and in differentiated populations. As a positive control, parallel quantification of Oct4 and Nanog, shows downregulation of these pluripotent markers upon embryoid body differentiation, showing a successful differentiation (**Figure 3-1**). The observation that Vgll4 increases throughout the differentiation timecourse means that it is upregulated upon differentiation. However, from this analysis it is not possible to determine whether it is upregulated in a subset of cells or throughout the entire population. Given that Vgll4 is a co-transcriptional regulator, its function in pluripotent and differentiated cells might be dictated by the interacting transcription factors present in that cell type.

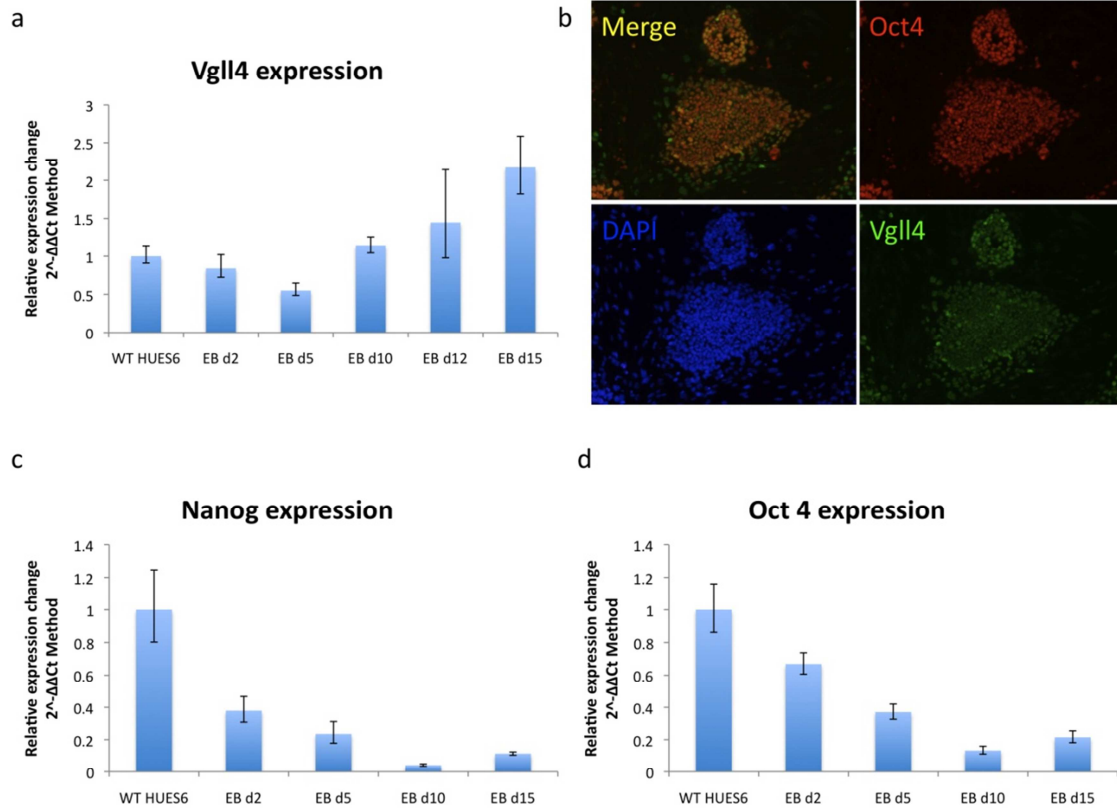


Figure 3-1. Vgl14 is expressed in pluripotent and differentiated cells. a) Expression of Vgl14 relative to GAPDH in pluripotent cells (WT HUES6) and across an embryoid body (EB) differentiation timecourse. The days of differentiation are indicated after “d” in the X axis. b) Immunostaining of Oct4 and Vgl14 in hES cells. Vgl14 is detected in the Oct4-positive and some Oct4-negative cells. c) Expression of Nanog relative to GAPDH in pluripotent cells and EBs. d) Expression of Oct4 relative to GAPDH in pluripotent cells and EBs.

3.2.2. Effects of Vgll4 overexpression at the global transcriptional level

To better understand the transcriptional changes taking place in hES cells overexpressing Vgll4, we compared the global transcriptional profiles of wildtype (WT) hES cells and hES cells overexpressing Vgll4 using Illumina microarrays. Arrays were then analyzed using SAM (Significance Analysis of Microarrays) to find genes that were significantly differentially expressed between the two groups [13]. The list of significant genes was analyzed using GeneGO for network and gene ontology information. The first analysis, based on the individual gene ontology categories, revealed that genes involved in cellular processes including development, morphogenesis, and response to stress responded significantly to Vgll4 overexpression. The cell processes along with the p-values for upregulated and downregulated genes are indicated in Table 1 (**Table 3-1**). The second analysis identified networks for the list of differentially regulated genes based on reported interactions. This analysis revealed a significant response in networks regulating apoptosis and cell adhesion (**Table 3-2**). In both of these tables the p-values represent the significance that the indicated process is affected based on the genes that are differentially regulated. It is worth noting that this analysis alone does not provide information about the overall outcome of the pathway, i.e., whether it is activated or inhibited. To address whether this indicated an activation or repression of certain pathways of interest, we assessed the change in expression of individual genes in these pathways. We noticed significant downregulation of genes acting as key positive regulators of the apoptosis pathway, for instance Caspase 9, Apoptotic Protease Activating Factor 1 (APAF1), some Tumor Necrotic Factor (TNF) receptors, and BCL-2-like 1 (BCL2L1) in the Vgll4 population. We also noticed a downregulation of multiple

cytoskeletal genes and various regulators of the Rock pathway, such as Ras homolog gene family, member B (RhoB), Rho Guanine nucleotide Exchange Factor 3 (ARHGEF3, an activator of Rho), and Arrestin beta 1 (ARRB1, a RhoA activator and mediator of stress fiber formation) (**Figure 3-2**). These changes are currently being verified by real-time PCR. The changes in these individual genes are worth noting, however, we are aware that in some cases the fold-change is small. Therefore from this analysis alone it is not possible to know whether these changes are biologically significant despite being numerically significant. Nevertheless, taken together, these analyses suggested a connection to the regulation of survival, possibly coupled with communication and contact to neighboring cells.

Table 3-1. Cellular processes with a significant response to Vgll4 overexpression in hES cells

#	Processes	pValue (upreg)	pValue (downreg)
1	system development	4.121E-3	9.859E-31
2	anatomical structure development	8.504E-4	2.441E-30
3	developmental process	9.794E-6	2.136E-28
4	multicellular organismal development	6.922E-6	3.975E-28
5	anatomical structure morphogenesis	1.921E-3	4.767E-26
6	organ development	5.178E-4	2.115E-22
7	vasculature development	4.849E-3	1.702E-19
8	tissue development	2.092E-3	2.038E-18
9	response to stress	6.062E-4	1.414E-17
10	regulation of cellular component organization	7.942E-4	4.408E-17

Table 3-2. Functional networks with a significant response to Vgll4 overexpression in hES cells

#	Networks	pValue (upreg)	pValue (downreg)
1	Cell adhesion_Integrins signaling to Beta-catenin	5.253E-1	2.576E-05
2	Development_Cytoskeleton biogenesis	7.800E-1	4.156E-05
3	Development_Cartilage development	3.355E-1	3.824E-04
4	Blood coagulation_Calpain system	5.162E-1	7.398E-04
5	Development_Ossification_BMPs	2.705E-1	9.927E-04
6	Transmission of nerve impulse_Ephrin receptors	6.135E-3	2.327E-03
7	Apoptosis_LIGHT, Apo-2, CD27BP, FasL, TWEAK, TNF-alpha signaling	2.221E-2	2.584E-03
8	Cell adhesion_PAI1 signaling, PPAR-beta (delta), RXR-alpha regulation	4.882E-1	2.584E-03
9	Apoptosis_NGF,TNF signaling,NF-kB regulation	2.380E-2	2.864E-03
10	Cell adhesion_Signaling to MLCP via Integrins	8.269E-1	3.493E-03

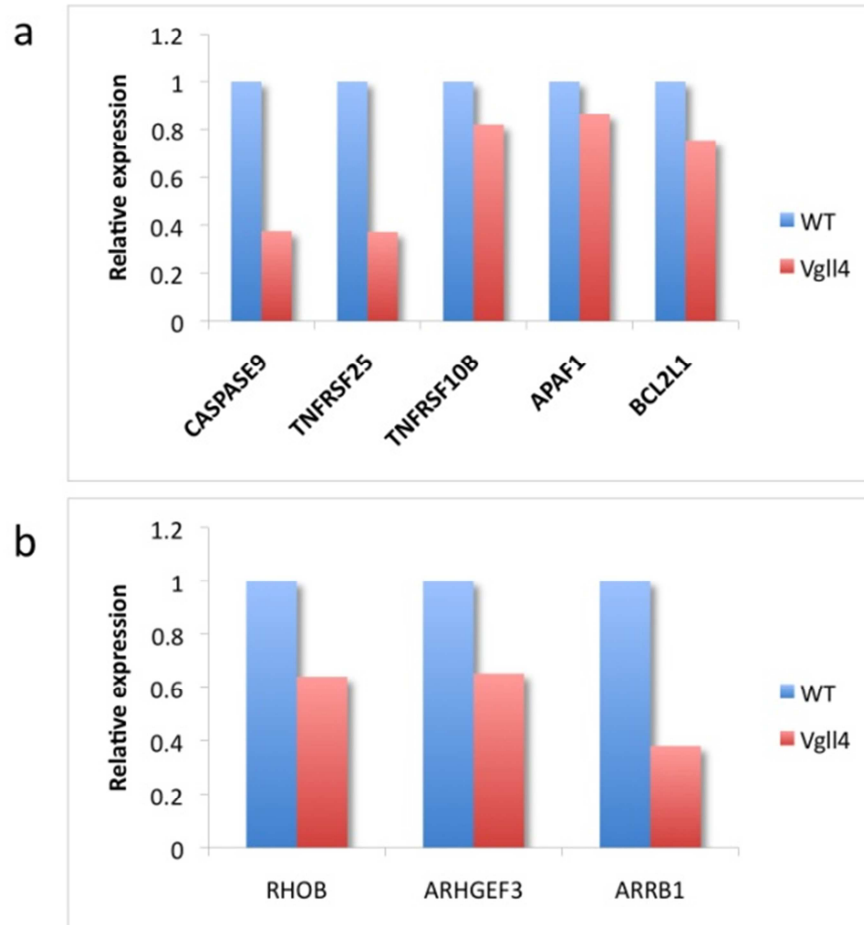


Figure 3-2. Vgll4 causes downregulation of genes involved in apoptosis and in Rho-Rock pathway activation. a) Graphical representation of microarray data showing the relative expression changes for pro-apoptotic genes in Vgll4 relative to WT hES cells. The q-values for these changes in expression are: Caspase 9 q = 7.29%; TNFRSF25 q = 7.67 %; TNFRSF10B q = 2.44%; APAF1 q = 3.35%; BCL2L1 = 4.61%. b) Graphical representation of microarray data showing the relative expression changes for genes involved in Rho-Rock signaling in Vgll4 relative to WT hES cells. The q-values for these changes in expression are: RHOB q = 2.34%, ARGEF3 q = 2.75%, ARRB1 q = 7.67%. Q-values were obtained by analysis with SAM.

3.3 Vgll4 overexpression results in faster doubling time of pluripotent cell populations

hES cell lines transduced with Nanog, Tubulin and Vgll4 were maintained over multiple passages. In this maintenance, Vgll4-hES cells consistently had 2-3 fold higher numbers of cells at the end of each passage despite the same number of cells being plated at the beginning. The exponential growth formula ($N = N_0 e^{kt}$) reveals a higher growth rate (k) in multiple hES cell and iPS cell lines overexpressing Vgll4, indicating that this effect is not cell-line specific (**Figure 3-3**).

The higher rate of population doubling in Vgll4-transduced cells could be explained through more than one mechanism. It could be due to an increase in survival, for instance a higher proportion of cells surviving after the initial plating or during the self-renewing conditions after initial plating. It could be caused by a faster replication rate of cells upon plating. It could be a combination of increased survival and proliferation. Our initial assessment of changes to cell cycle dynamics is presented in Chapter 4.

Previous gene expression analyses revealed an important role for Vgll4 in the regulation of stress and cell death pathways as well as changes in multiple cytoskeleton and cell adhesion proteins, suggesting a role for Vgll4 in regulating attachment to the substrate and neighboring cells. Human ES cells are particularly sensitive to dissociation [8, 9, 11, 14], therefore, we began by exploring the question of whether Vgll4 modulates survival in hES cells in response to dissociation.

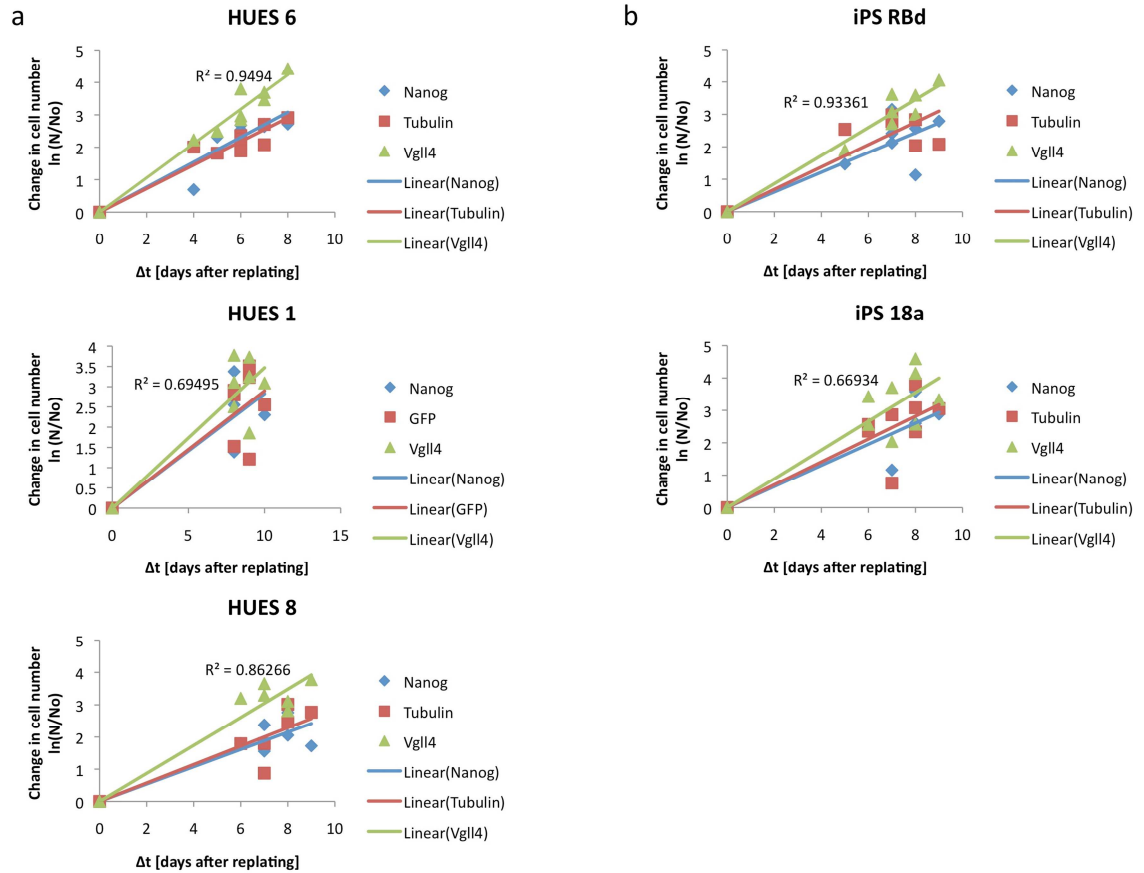


Figure 3-3. Pluripotent cells overexpressing Vgll4 have a higher population-doubling rate in maintenance conditions. Graphs depicting the growth rate (k) using the exponential growth formula $N = N_0 e^{kt}$ where N = final number of cells, N_0 = initial number of cells, t = time after plating in days. The data was plotted on a log-normal scale and a linear fit was performed. a) Human embryonic stem cell lines overexpressing Nanog, Tubulin, or Vgll4 and maintained in self-renewing conditions. Eight timepoints were analyzed for HUES6, seven for HUES1, and six for HUES8. b) Human induced pluripotent stem cell lines overexpressing Nanog, Tubulin, or Vgll4 and maintained in self-renewing conditions. Seven timepoints were analyzed for iPS RBd and eight for iPS 18a.

3.4 Vgll4 protects pluripotent cells from dissociation stress

3.4.1. *Vgll4 maintains a higher proportion of live cells*

Disrupting cell-to-cell contact in hES cells results in myosin hyperactivation and apoptosis [8, 12]. Low survival of hES cells upon passaging and dissociation poses a difficulty for certain applications of these cells, including genetic manipulation to generate reporter lines and correct disease-related genetic mutations. Therefore, we set out to answer whether Vgll4 had a role in promoting the survival of hES cells upon dissociation. We took advantage of changes in membrane asymmetry and permeability -one of the hallmarks of apoptosis- to identify the proportion of live cells, apoptotic cells, and dead cells after treatment with dissociation agents.

To disrupt cell-to-cell interactions without affecting membrane-bound proteins and receptors, a Hank's-based enzyme-free dissociation buffer was used at a 1:5 dilution with hES cell medium. As an alternative treatment, Ethylenediaminetetraacetic acid (EDTA) was added to hES cell medium at a concentration of 500 μ M to disrupt Calcium-ion-dependent E-cadherin junctions. Etoposide, a topoisomerase inhibitor, is known to cause apoptosis by introducing double-strand DNA breaks [15, 16]. We used etoposide at a concentration of 10 μ M a positive control for apoptosis. We expected hES cells to be especially sensitive to this treatment, as etoposide efficiently affects rapidly dividing cells [17]. Untreated cells were fed normally with hES medium.

After 12-18 hours, both floating cells and attached cells were harvested with 0.05% Trypsin-EDTA for 3 minutes and stained with Allophycocyanin (APC)-conjugated Annexin V and Propidium iodide (PI). Annexin V detects asymmetrical and

compromised membranes by binding to phosphatidylserine, a phospholipid in the plasma membrane that localizes to the cytosolic leaflet of the plasma membrane in live but not apoptotic cells [18]. PI detects DNA but can only enter the cell when membranes are compromised, as happens in dead cells. Together, these reagents allow the identification of live, apoptotic, and dead cell populations by flow cytometry. A representative example of these results is depicted in Figure 3 (**Figure 3-4**).

We found that Vgll4 had a statistically significant effect in maintaining a higher proportion of live cells. Vgll4 allowed the maintenance of close to 70% of live cells compared to the Tubulin control with approximately 50% in untreated conditions. This represents a 40% increase in the proportion of live cells compared to the Tubulin control. Additionally, Vgll4 cells maintained over 56% live cells versus 43% in Tubulin cells after EDTA treatment. This represents an increase of 30% in the proportion of live cells relative to Tubulin controls. In addition to maintaining a higher proportion of live cells, Vgll4 was also able to decrease the proportion of apoptotic cells in both conditions, with 37-53% fewer apoptotic cells. These results demonstrate a powerful effect in counteracting cell death caused by cell dissociation (**Figure 3-5**) and can contribute to our observation of a higher population-doubling rate. It is worth noting that for these analyses both floating and attached cells were assayed after treatment, which explains the lower proportion of live cells compared to assaying the adherent cells alone. Cells that remain adherent in untreated conditions are consistently over 90% live.

Interestingly, the proportion of dead cells in Vgll4 cells is consistently higher after treatment with etoposide. Etoposide is known to efficiently target rapidly dividing cells [17]. Therefore, it is possible that this is caused by an increased rate of replication in Vgll4 cells that our cell cycle analyses have not yet uncovered.

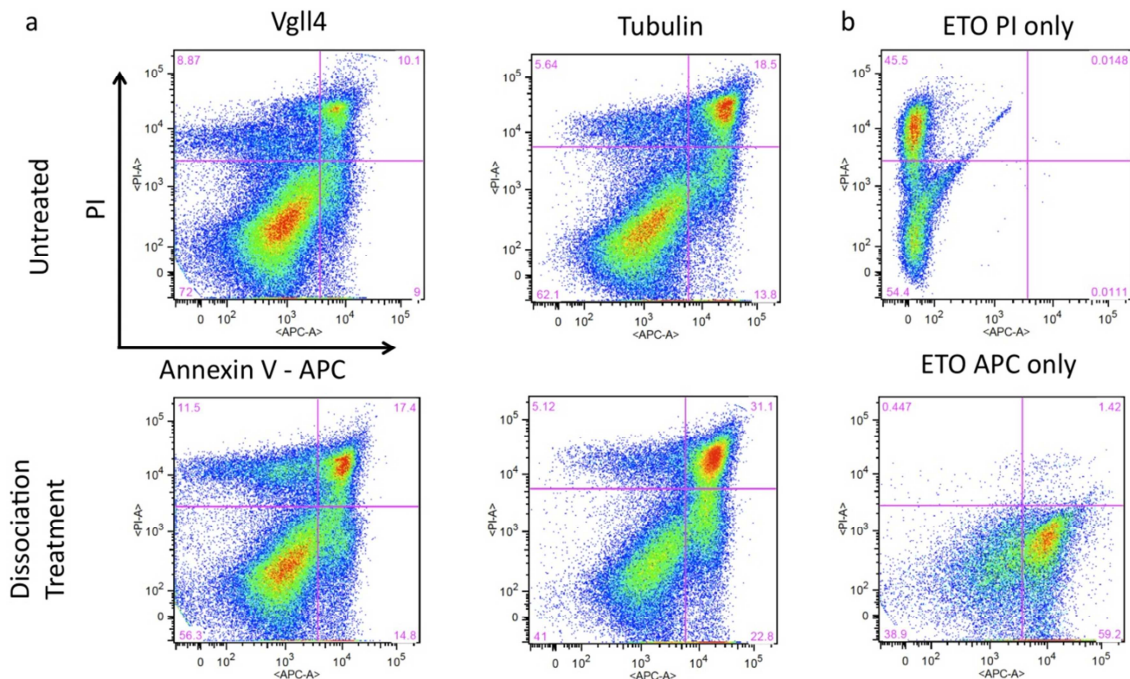


Figure 3-4. Annexin V and PI staining can be used to detect live, apoptotic, and dead cell populations in hES cells. a) Vgll4- and Tubulin-transduced hES cells either untreated or treated with dissociation buffer for 12-18 hours and stained with Annexin V-APC and PI, then analyzed by FACS. Live cells are APC⁻, PI⁻; apoptotic cells are APC⁺, PI⁻, and dead cells are PI⁺. b) Etoposide (ETO)-treated single-color controls for APC and PI are used to accurately set gates for the experiment.

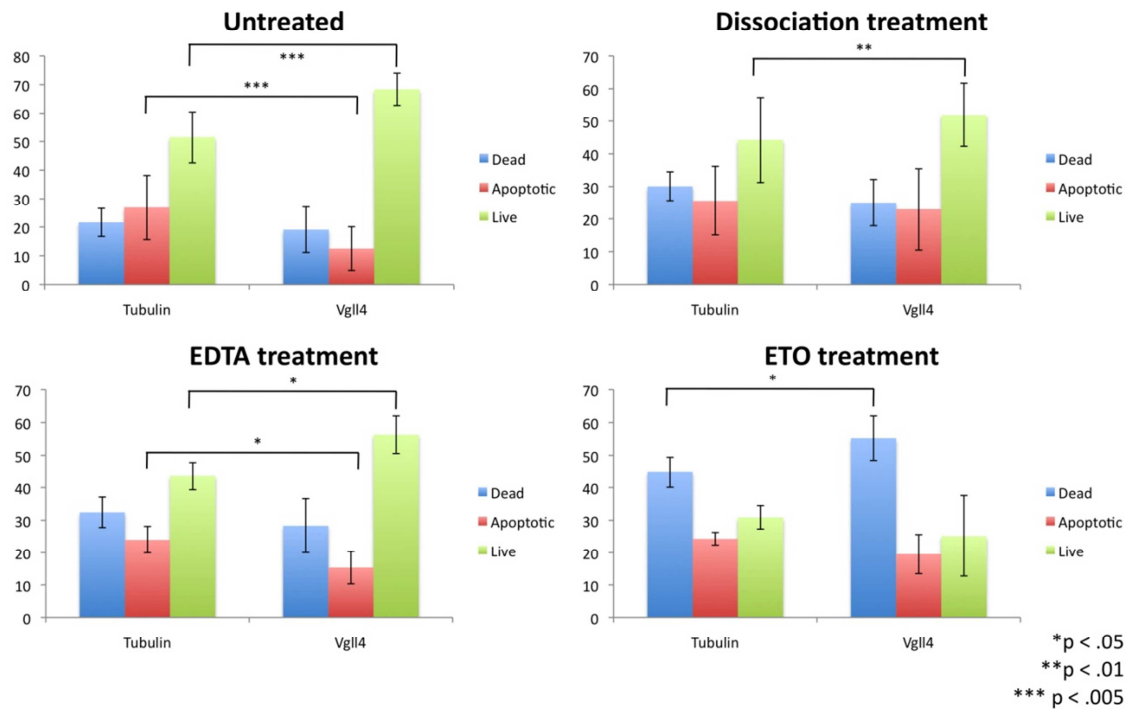


Figure 3-5. Vgll4 promotes survival and inhibits apoptosis in response to cell dissociation. hES cells overexpressing Vgll4 or Tubulin were treated with a Hank's-based enzyme-free dissociation buffer or 500 μ M EDTA or 10 μ M etoposide (ETO) for 12-18 hours. The proportion of live, apoptotic, and dead cells was assessed by Annexin V and PI staining. Results represent flow cytometry data from 6 independent experiments. Error bars represent the standard deviation and the p-values were obtained with an unpaired Student's T-test.

3.4.2. Vgll4 suppresses Caspase activity

We wanted to assess whether the increased proportion of live cells and decreased proportion of apoptotic cells in Vgll4-transduced cells resulted from a decrease in caspase activity. Caspase-9 is an initiator caspase that associates with APAF-1 to form the apoptosome complex, which activates effector caspases. Caspases 3 and 7 are effector caspases that cleave multiple substrates, leading to apoptosis and eventually cell death [19-21].

Following the same treatment explained above we used a luminescence-based assay to assess Caspase-3/7 and Caspase-9 activity. This assay uses a proluminescent caspase substrate that serves as a substrate for luciferase upon cleavage by caspases. Vgll4-hES cells showed a significant decrease in activated Caspase-3/7 and 9 activity in both the untreated and dissociation conditions (**Figure 3-6**). This suggests that Vgll4-hES cells have a lower basal activation of caspases, perhaps resulting in a protective effect against certain stress stimuli.

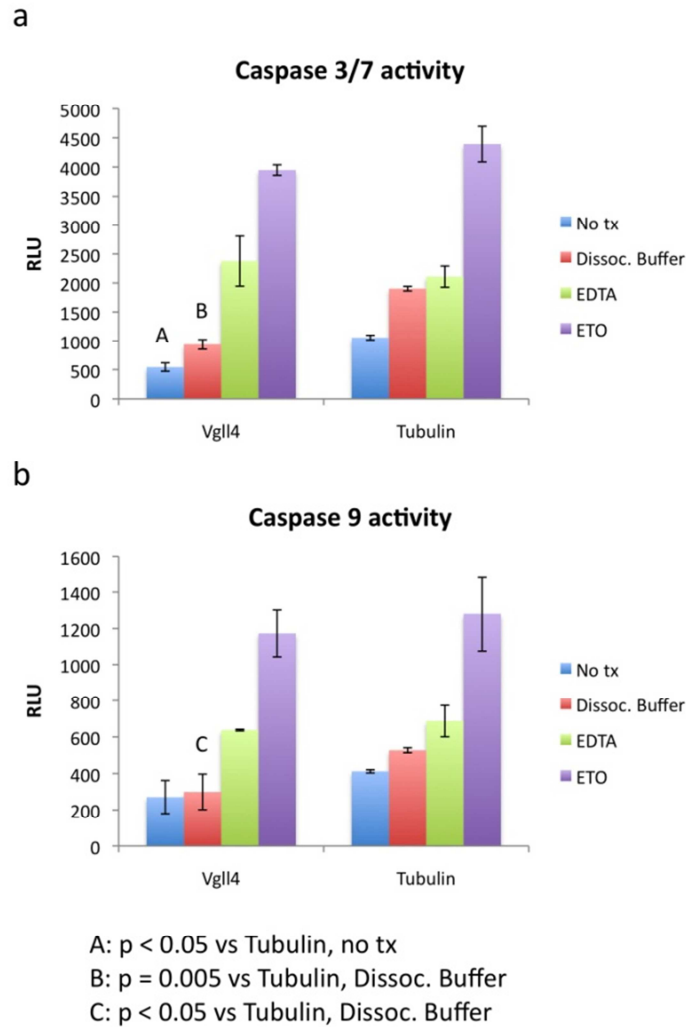


Figure 3-6. Vgll4 decreases Caspase 3/7 and Caspase 9 activity. a) Caspase 3/7 and b) Caspase 9 activity for Vgll4- and Tubulin-hES cells after treatment with dissociation conditions. Both were assessed using caspase luminescence assays. Relative luminescence units (RLU) represent the signal after subtracting the background. Background was obtained by mixing medium with assay buffer. Error bars represent the standard deviation of triplicates. P-values were obtained using an unpaired Student's T-test.

3.4.3. *Vgll4* is necessary to regulate survival and caspase activity of hES cells

A. Identification of an shRNA against *Vgll4*

We wanted to test whether *Vgll4* was necessary to regulate survival in hES cells. To this end, single-stranded oligonucleotides were obtained from the Invitrogen database and annealed as instructed. The hairpins were then cloned into the same pHAGE vector used for the rest of our stably transduced lines and the population of cells that was successfully transduced was selected using puromycin. We identified a short-hairpin RNA (shRNA) against *Vgll4*, referred to as V78. Transduction of hES cells with V78 resulted in a 60% knockdown of *Vgll4* compared with cells transduced with a scrambled control (mir neg) as assessed by real-time PCR (**Figure 3-7**). We are currently optimizing conditions for the Western Blot verification of these results.

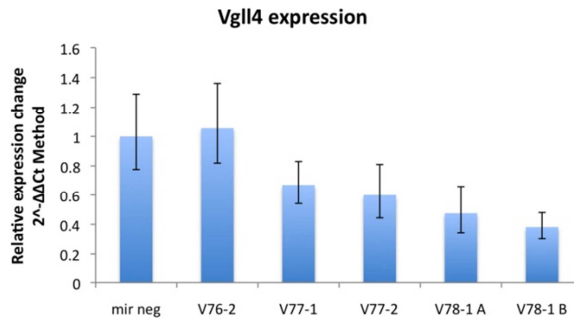


Figure 3-7. V78 is a shRNA that achieves 60% knockdown of *Vgll4*. *Vgll4* expression levels relative to GAPDH in cells transduced with a shRNA against *Vgll4* (V78) or a scrambled control (mir neg). V78-1 A and B represent different virus production batches and transductions.

B. Assessment of live cell proportion and Caspase activity

Cells stably expressing a scrambled control (mir neg), V78, or Vgll4 itself were treated with the dissociation conditions described earlier. Annexin V and PI staining were used to assess the proportion of live, apoptotic, and dead cells in these populations. We found that knockdown of Vgll4 increased the proportion of dead and apoptotic cells and decreased the proportion of live cells. This effect was especially noticeable under dissociation conditions (**Figure 3-8**). Our statistical analysis identified a significant difference when V78 cells were compared to Vgll4 but not mir neg. One explanation for this finding is that the change in proportions is not numerically significant; however, this could still have a biological effect, albeit subtle. The second possibility is that there is still enough residual Vgll4 activity and a stronger knockdown of is necessary to observe a significant change. Additionally, using the different assays we have introduced earlier should give a more complete picture for the consequences of decreased Vgll4 levels. For instance, we are currently testing the effect of Vgll4 knockdown in population-doubling rate and colony-forming ability.

We also tested whether knockdown of Vgll4 would affect Caspase activity. For this purpose, we treated cells with dissociation conditions and assessed the activation of Caspase 3/7 and Caspase 9 with a luminescence-based assay. V78 caused a small but statistically significant increase in Caspase 3/7 and 9 activities under dissociation conditions (**Figure 3-9**).

A: $p = 0.005$ vs Vgll4, dead
 B: $p < 0.05$ vs Vgll4, apoptotic
 C: $p < 0.05$ vs Vgll4, live
 D: $p < 0.005$ vs Vgll4, apoptotic
 E: $p < 0.05$ vs Vgll4, dead

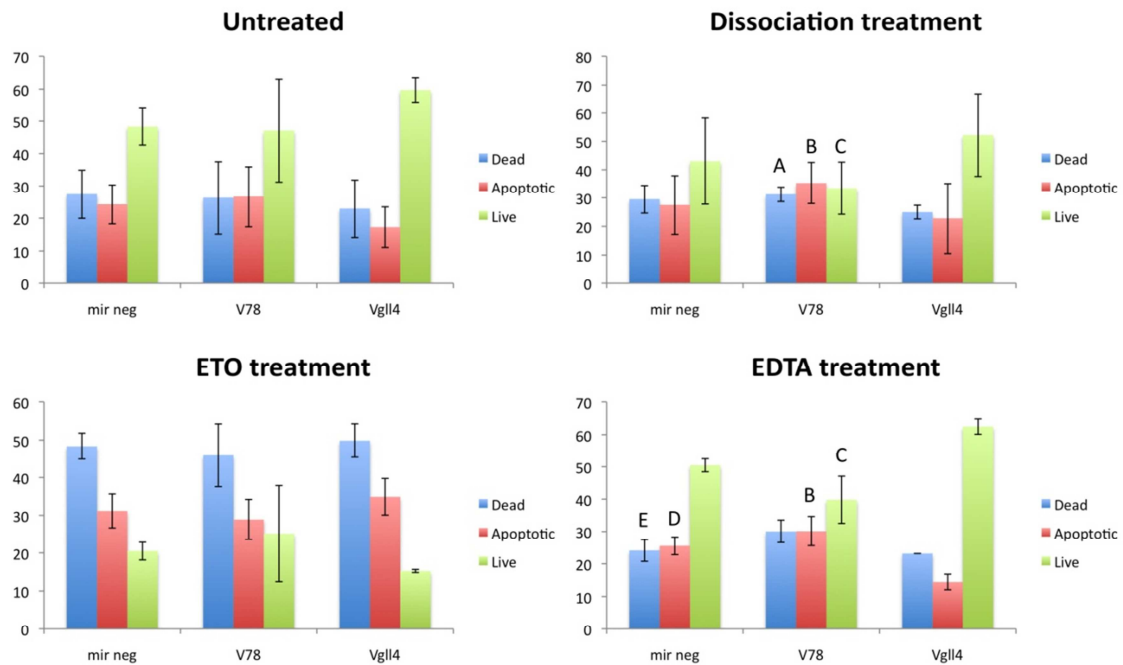


Figure 3-8. Vgll4 knockdown increases hES cell sensitivity to dissociation conditions. hES cells stably expressing a scrambled control (mir neg), V78, or Vgll4 were treated with a Hank's-based enzyme-free dissociation buffer, 500 μ M EDTA or 10 μ M etoposide (ETO) for 12-18 hours. The proportion of live, apoptotic, and dead cells was assessed by Annexin V and PI staining. Results represent flow cytometry data from 3 independent experiments. Error bars represent the standard deviation and the p-values were obtained with an unpaired Student's T-test.

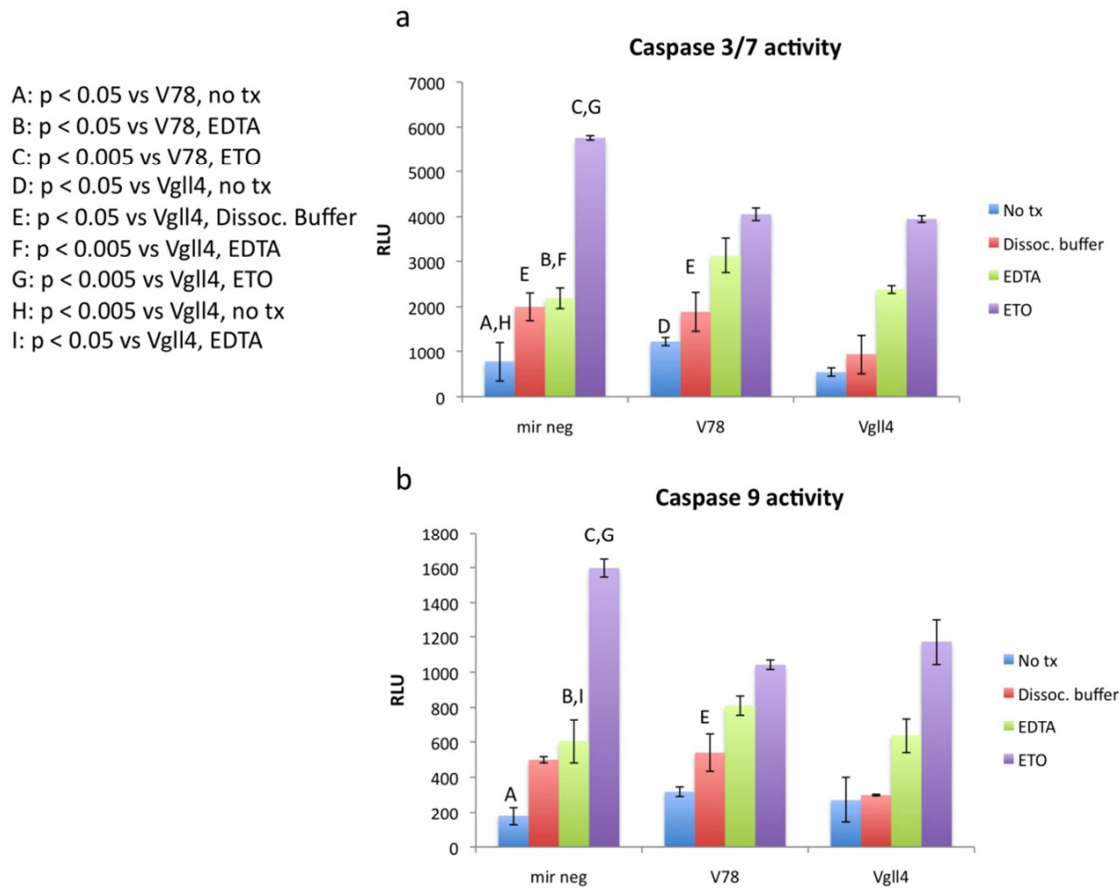


Figure 3-9. Vgll4 knockdown increases caspase activity upon treatment with dissociation conditions. a) Caspase 3/7 and b) Caspase 9 activity for mir neg, V78, and Vgll4-hES cells after treatment with dissociation conditions. Both were assessed using Caspase-Glo luminescence assays. Relative luminescence units (RLU) represent the signal after subtracting the background. Error bars represent the standard deviation from triplicate samples. P-values were calculated using an unpaired Student's T-test.

3.5 Vgll4 confers an advantage in colony forming potential from single cells

The colony-formation assay has historically been used as a test for self-renewal of multipotent progenitors. In the case of hES cells, it is also a stringent assay for survival in response to dissociation. We wanted to directly test the ability of Vgll4-hES cells to form colonies after dissociation into single cells. Nanog, GFP, and Vgll4-hES cells were dissociated using 0.05% Trypsin-EDTA for 3 minutes at room temperature and 1000, 100, 10 or 1 cell per well were sorted using FACS onto 96-well plates using an antibody recognizing Tra1-60 as a marker to selectively sort out only pluripotent cells. After 10 days, the cells were fixed, stained for DAPI and scored for the presence or absence of a colony in a well. Vgll4 increases hES cell colony-forming efficiency by 2-3 fold in the presence and absence of Rock-inhibitor (**Figure 3-10**). We observed a consistent increase in the colony forming ability of Vgll4 cells. However, the variance in the number of colonies prevented us from performing statistical analysis.

In addition, to assess whether the clonally-derived cells had remained undifferentiated during the 10 days in culture. To this end, colonies were stained with pluripotency markers. In addition, the cells retained expression of the lentiviral gene as assessed by GFP or HA expression (**Figure 3-11**).

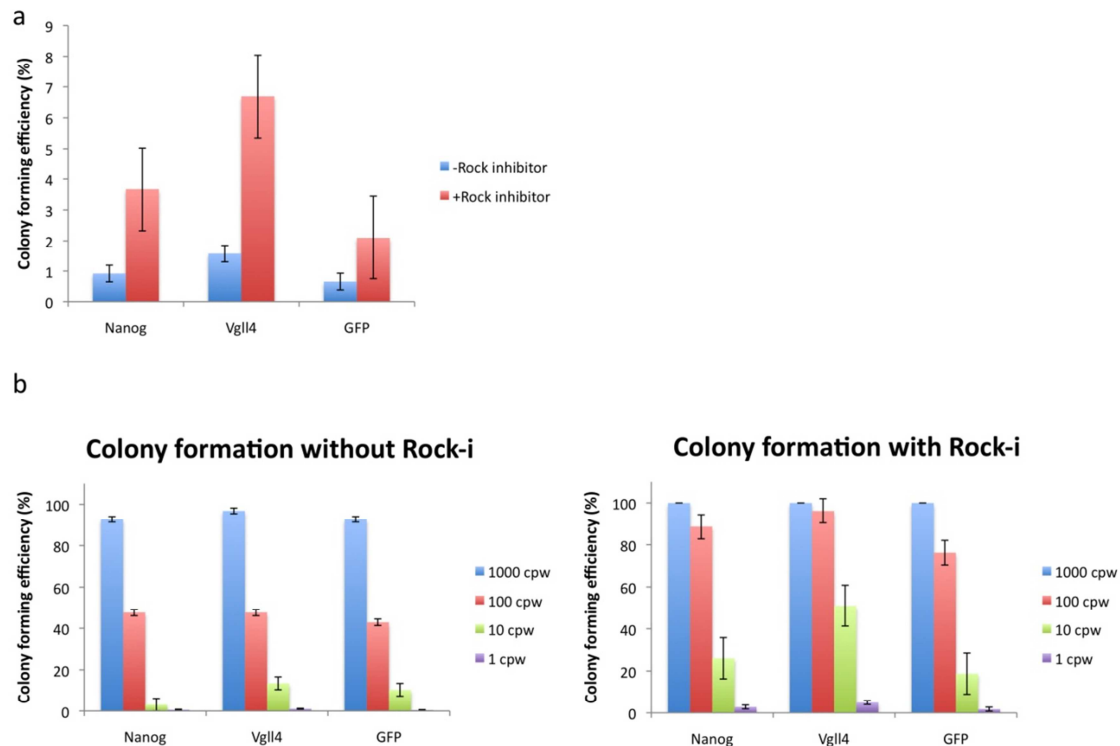


Figure 3-10. Vgll4 increases the colony-forming efficiency of hES cells. a) hESCs transduced with Nanog, GFP or Vgll4 were plated at single cell densities on 96-well plates and counted 10 days after plating. Percentage of wells where colonies formed is shown for conditions with and without Rock inhibitor. b) Results for low-density plating at 1000, 100, 10 and 1 cells per well. Errors bars represent the standard error from 2 experiments.

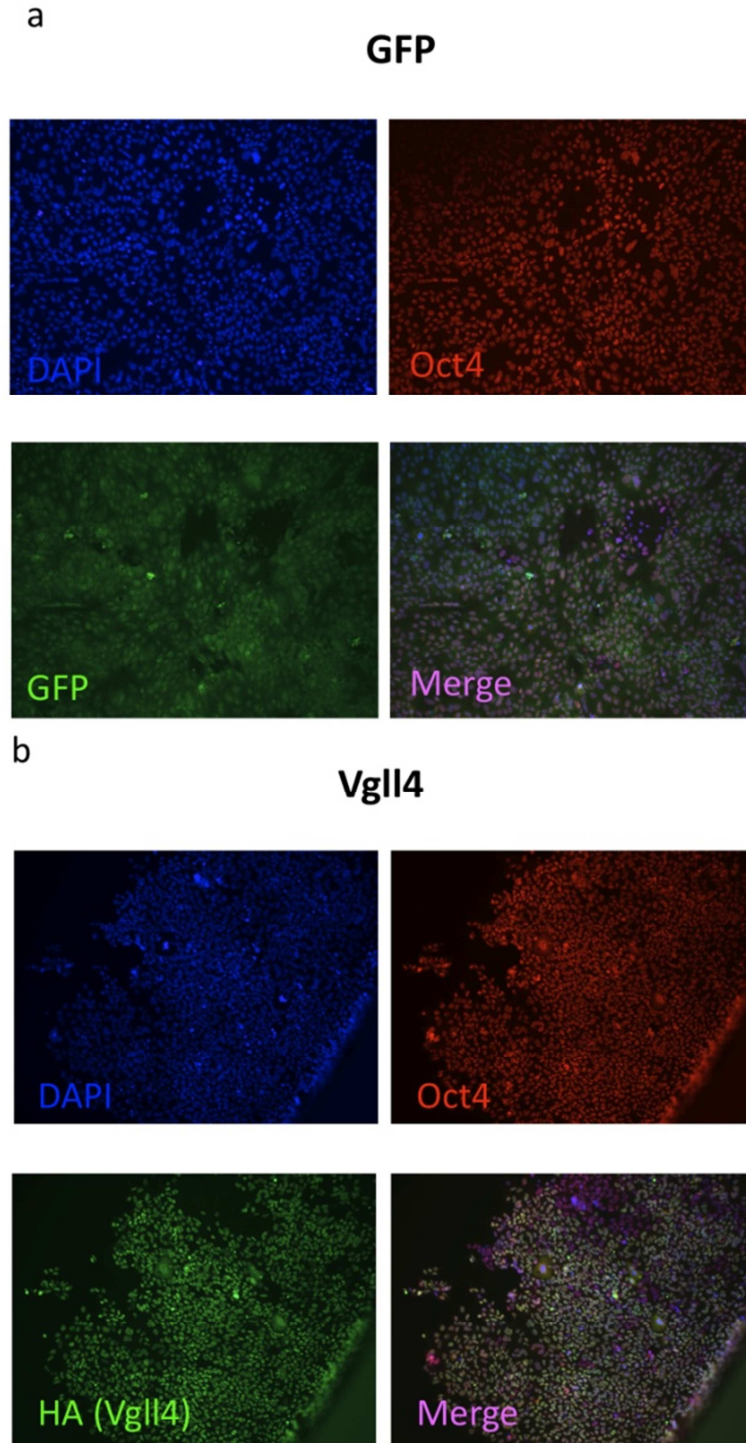


Figure 3-11. Clonally-derived cells maintain expression of pluripotency markers and lentiviral genes. After 10 days in culture, colonies derived from single cells were fixed and analyzed for the expression of the pluripotency marker Oct4 and the lentivirus-encoded GFP (a) or Vgl14-HA (b).

3.6 A possible interaction between Vgll4 and the Rho/Rock pathway for promoting hES cell survival

Finally we addressed whether the activity of Vgll4 was required for the beneficial effects of Rock inhibition on hES cell survival. A dose-response curve for Rock inhibition showed an increased colony forming efficiency at increasing concentrations of the inhibitor with a plateau reached at concentrations between 6-10 μM (Figure 3-12 a). We next compared the effect of increasing concentrations of the inhibitor on cells deficient for Vgll4 using hES cells transduced with V78. No significant differences were detected between control and knock-down cells in a colony forming assay either in the absence of Rock inhibitor or at high concentrations of the inhibitor. However, at intermediate concentrations of Rock inhibitor (1 μM -7.5 μM), we observed that cells deficient for Vgll4 have a decreased colony-forming efficiency compared to wildtype cells (Figure 3-12 b,c). These results demonstrate that Vgll4 may be necessary to fully benefit from the inhibition of Rock activity, therefore implying a possible genetic interaction between Vgll4 and the Rock-signaling pathway for maintaining hES cell survival.

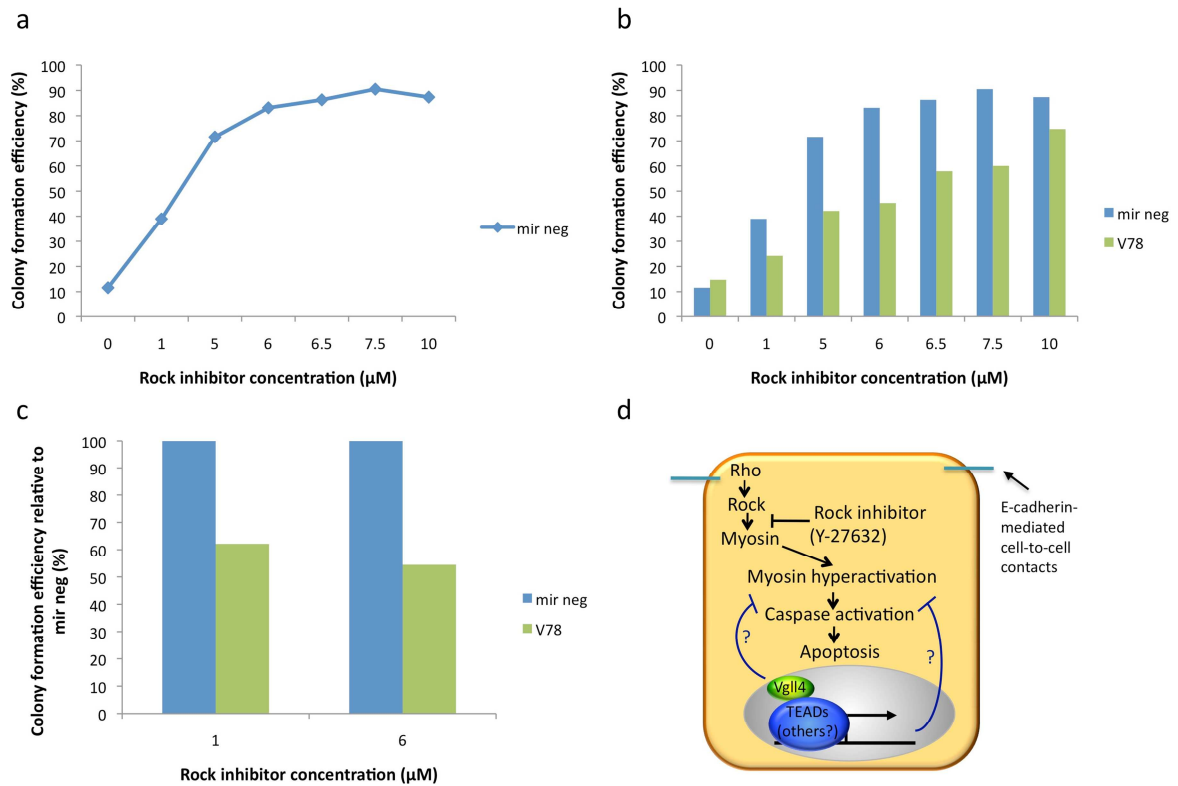


Figure 3-12. A possible interaction between Vgll4 and the Rho/Rock pathway promotes survival of hES cells. Colony formation efficiency for 100 cells/well treated with the indicated concentrations of Rock inhibitor. a) Rock inhibitor dosage response curve in control cells (mir neg). b) Colony formation efficiency of control (mir neg) and Vgll4-knock-down (V78) cells. V78 are deficient in their colony-forming ability. c) Full Vgll4 functionality is required to benefit from Rock-inhibition. V78 colony-forming efficiency is shown normalized to the colony-forming efficiency of mir neg. Increasing concentrations of Rock inhibitor are unable to rescue for Vgll4-deficiency at non-saturating concentrations. d) Model for mode of action of Vgll4. Rho signaling is activated upon disruption of E-cadherin cell-to-cell contacts resulting in apoptosis. Vgll4 could inhibit cell death by modulating the expression of apoptosis and cytoskeleton genes. Alternatively, Vgll4 could have a role in the cytoplasm independent from its role as a co-transcriptional regulator in the nucleus.

3.7 Conclusions and Discussion

Vgll4 has not been described previously in the context of hES cells. Our findings identify Vgll4 as a novel gene promoting the survival of hES cells in response to dissociation. Its pro-survival activity has important implications for overcoming the problem of low survival of hES cells in clonal densities, which would greatly facilitate the derivation of reporter lines and the ability to correct disease-related mutations. Both of these would represent a step forward in disease-modeling and therapy using pluripotent cells.

Interestingly, Vgll4 is expressed in both self-renewing hES cells and differentiated cells. Nevertheless, the specificity of its activity in pluripotent cells could be dictated by two factors: 1) the specific transcription factors present at each cell stage, thus leading to regulation of a different set of genes in hES cells versus differentiated cells, and 2) the particular characteristics, such as the transcriptome and the epigenome of each cell type. For instance, sensitivity to dissociation is an important consideration for hES cells but not for other cell populations tested such as normal human dermal fibroblasts and human vascular endothelial cells.

Global transcriptional analysis in hES cells revealed significant differences in cellular processes including organogenesis and response to stress through the modulation of apoptosis and cytoskeletal genes in cells transduced with Vgll4. Together with our Annexin V/PI data, this suggests that Vgll4 is a mediator of apoptosis. Given the current understanding of the role of dissociation and its connection to the myosin cytoskeleton and hES cell apoptosis [8, 14], this could suggest a link between changes to

cytoskeleton genes and increased survival in Vgll4 cells. For instance, Vgll4 could have a role in allowing hES cells to attach more efficiently to the substrate or neighboring cells through cytoskeletal changes while inhibiting members of the apoptosis pathway that unleash a cell death response. A lower rate of cell death in response to dissociation could also contribute to the faster population-doubling rate that we observed in multiple pluripotent cell lines. It is important to note that Vgll4 is a complex gene with multiple predicted splice variants. Therefore, it is possible that it could also modulate other mechanisms that contribute to this observation, for instance, cell cycle dynamics. We initially hypothesized that the observed increase in the rate of population-doubling was due to an increase in cell proliferation. Our initial experiments, presented in Chapter 4, show no difference in the distribution of cells in the cell cycle or in the proportion of mitotic cells. We refer the reader to Chapter 4 for an explanation of our future directions in testing for changes to the cell cycle. The expression pattern and function of the different splice variants is currently not known. The ORF contained in the human ORFeome represents isoform a, which has the longest transcript and gives rise to the longest protein.

Our results show that Vgll4 functionally contributes to an increase in the proportion of live cells and a decrease in the proportion of apoptotic cells in untreated conditions and after dissociation treatment. These observations could be explained, at least in part, by a decreased activity of initiator and effector caspases. These results could have functional and therapeutic relevance for improving maintenance and subcloning strategies for pluripotent stem cells. To evaluate if the increase in proportion of live cells in untreated conditions is enough to explain the difference in

doubling time, it would be possible to explore the differences in cell number early after plating. This assay is currently in progress.

A decrease in Vgll4 levels resulted in an increase in apoptotic cells and a decrease in live cells, as well as an increase in caspase activity under dissociation conditions, indicating a necessary role for regulating caspase activation upon dissociation stress. However, the effect of knockdown of Vgll4 on the levels of activated Caspases was smaller than expected in untreated conditions. This might also explain the absence of a significant change in the proportion of Annexin V and PI-positive populations in untreated conditions. Some explanations for this observation include the fact that this is a mild knockdown; effect of Vgll4 downregulation could be more significant with a more complete knockdown but we were unable to identify a shRNA that led to a more than 60% reduction in the levels of Vgll4 RNA. A second explanation is that redundancy is likely to exist in the regulation of cell survival. Many mechanisms and pathways orchestrate the decision of whether a cell lives or dies. It is possible that other members of this pathway can rescue the absence of one gene. For this reason, finding an effect through a gain-of-function approach is more feasible than through a loss-of-function approach. A third explanation is that specific conditions are needed to unmask the Vgll4 knock-down phenotype. For this purpose, we tested the colony-formation efficiency of Vgll4-deficient hES cells at increasing concentrations of Rock inhibitor and found that these cells were impaired in their colony-forming ability. This result implies that normal Vgll4 levels are required to benefit from the effect of Rock inhibitor and suggest an interaction between these pathways to promote hES cell survival.

The discovery of Rock inhibitor and its ability to increase the survival of dissociated hES cells provided an important tool to begin to address the problem of low viability of hES cells upon dissociation [9]. However, survival remains a challenge for many applications of pluripotent cells. Importantly, Vgll4 is able to increase the colony-forming ability of single hES cells by 2-3 fold in the presence and absence of Rock inhibitor. Given that Vgll4 is a co-transcriptional regulator, we hypothesize that Vgll4 may act in the nucleus to mediate transcriptional changes that prevent activation of apoptosis. Alternatively, Vgll4 could be exported out of the nucleus to carry out a transcription-independent role for modulating cell survival, perhaps by directly interacting with members of the Rock pathway in the cytoplasm (Figure 3-12 d). Therefore, this study contributes to a deeper understanding of the mechanisms controlling cell death and represents a possibility to uncover new chemicals to make survival more efficient.

3.8 Methods used in this chapter

3.8.1. Culture of hES cells

HUES and iPS lines were plated on irradiated murine embryonic fibroblasts (MEFs) and grown in media containing KO-DMEM (Invitrogen), 10% Knockout Serum Replacement (KOSR, GIBCO), 10% human plasma fraction (Talecris), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (GIBCO), .055 mM β -Mercaptoethanol (GIBCO), and 10 ng/ml bFGF (Invitrogen). Cultures were passaged using 0.05% Trypsin-EDTA (GIBCO) at a ratio of 1:6-1:10 every 5-7 days.

MEFs were plated on plates coated with 0.1% gelatin. MEF media contained 1X DMEM (Mediatech), 10% FBS, 2mM L-glutamine (Invitrogen), and 0.1mM non-essential amino acids (GIBCO).

3.8.2. Embryoid body differentiation

hESCs transduced with the indicated genes were gently trypsinized and placed in a low-attachment 6-well dish at a concentration of 1×10^6 cells/well in hES cell medium without bFGF. Cells were left undisturbed for the first 96 hours to allow for formation of individual EBs and media was changed every 48 hours thereafter. A fraction of EBs were collected at the indicated times and used as a source of RNA.

3.8.3. Real-time PCR analysis

RNA was harvested using a Qiagen RNeasy kit. DNase I treatment was used as indicated by the manufacturer to eliminate genomic DNA. Purified RNA was used as input for the reverse transcription reaction using the Superscript III First Strand kit

(Invitrogen). TaqMan assays (Applied Biosystems) were used for the following human genes and samples were prepared following manufacturer instructions: POU5F1, NANOG, VGLL4. GAPDH was used as a control. All samples were analyzed using a 7900HT machine (Applied Biosystems) using the Fast protocol as specified by the manufacturer (Applied Biosystems). Cycling parameters were the following: UNG incubation, 2 minutes at 50°C; Polymerase activation, 20 seconds at 95°C; denaturing, 1 second at 95°C; anneal/extend, 20 seconds at 60°C with 40 cycles of denaturing/extending/annealing. Resulting Ct values were then processed using the $\Delta\Delta C_t$ method to obtain relative changes in expression.

3.8.4. Microarray analysis

RNA was purified from samples in quadruplicates (biological replicates) using a Qiagen RNeasy kit and 200 ng of starting material were used as input for the Illumina TotalPrep Amplification Kit. Samples were then hybridized to an Illumina microarray. A selection of genes with a p-value < 0.05 on at least 5 arrays was analyzed using SAM (<http://www-stat.stanford.edu/~tibs/SAM>) with an FDR=7%. Network and gene ontology analyses were performed using GeneGO (<http://www.genego.com>).

3.8.5. Growth curve analysis

Cells were passaged as described above. An aliquot of cell suspension was counted using a Vi-Cell XR counter (Beckman Coulter). The exponential growth formula ($N = N_0 * e^{k\Delta t}$) was used to determine the growth rate (k).

3.8.6. Apoptosis analysis by Annexin V and PI

Cells were treated with a mixture of Enzyme-free dissociation buffer (Millipore) and hES medium at a ratio of 1:5, 500 μ M EDTA, or 10 μ M etoposide for 18 hours at 37°C and 5% CO₂. All floating cells were collected. Remaining adherent cells were then trypsinized and washed with ice cold PBS. Cells were stained following manufacturer instructions (Invitrogen) as follows. First, cells were resuspended in Annexin V binding buffer and aliquoted where necessary to have 1-2 x 10⁶ cells per sample of 100 μ l. Cells were then stained with 1 μ g/ml PI and 5 μ l of APC Annexin V. Samples were incubated at room temperature for 15 minutes protected from light. After the incubation period, cells were resuspended in 400 μ l of binding buffer and analyzed by FACS in a BD LSR II machine. Unstained and single-color controls were used to perform compensation and set gates.

3.8.7. shRNA knockdown

ssDNA oligos were obtained from Invitrogen for Vgll4 (V76: Hmi423276; V77: Hmi423277; V78: Hmi423278) and processed using the instructions for the BLOCK-iT Pol II miR RNAi Expression Vector Kits. shRNAs were cloned into the EF1 α -pDEST using Gateway cloning to use the same vector used in our screening strategy. Sequences were verified using Geneious. Lentiviral particles were made as outlined in Chapter 2. Cells were transduced with the lentiviral shRNA constructs as indicated in the transduction section and selected with puromycin. Cells were maintained in hESC medium for 7 days after selection. After this timepoint cells were collected for RNA analysis or maintained as described above for subsequent analyses.

3.8.8.hES cell transduction

Cells were MEF-depleted for 30-45 min on gelatin-coated plates as described previously. Cells were then counted and mixed with harvested lentivirus at an MOI of 2. Cells were incubated with the virus in a low-attachment dish for 2-3 hours at 37°C and 5% CO₂ with occasional rocking. After this time, the cells were pelleted by centrifugation at 1000 rpm for 5 minutes at room temperature. The cells were then plated onto Puromycin-resistant MEFs. 48 hours later, transduced cells were selected with 2 µg/ml of puromycin for 2 days, generating a population of >98% transduced cells.

3.8.9.Clonality assay

Cells were dissociated with 0.05% Trypsin-EDTA as described above for passaging. Cells were washed once in PBS and then incubated in 100 µl of a 1:100 dilution of mouse IgM anti-Tra 1-60 (Millipore) or rat IgM anti-SSEA-3 (Santa Cruz Biotechnology) in FACS buffer (2% Hyclone Fetal Calf Serum in PBS). Cells were incubated in primary antibody for 15-30 minutes on ice. After this incubation, cells were washed with PBS and stained with secondary antibodies conjugated to APC (Jackson ImmunoResearch) at a dilution of 1:300 for 15 minutes on ice. Cells were washed with PBS and resuspended in FACS buffer. Cells were filtered immediately before sorting through a 35 µm filter. A BD Aria was used for sorting. Cells were sorted onto 96-well plates previously coated with BD Matrigel for hESCs and containing 100 µl of MEF-conditioned media supplemented with 16 ng/ml of bFGF. As indicated, 1000, 100, 10, or 1 cell per well were plated. Rock inhibitor was used in the indicated wells at

a final concentration of 10 μ M. Media was changed every third day. After 10 days, cells were fixed with 4% PFA and stained with DAPI at 1 μ g/ml. Each well was inspected for the presence of colonies. A 4-cell minimum was considered for calling a colony. Immunofluorescence was performed on positive wells using a goat anti-Oct4 or a rabbit anti-HA as described below.

3.8.10. Immunofluorescent staining

Cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Two washes were performed after fixation. Cells were blocked for 1 hour at room temperature or overnight at 4°C in 5% donkey serum in 0.1% PBST (0.1% TritonX-100 in PBS). Cells were incubated with primary antibodies in block for 2 hours at room temperature or overnight at 4°C. Primary antibodies used are: goat anti-Oct-4, 1:200 (Santa Cruz biotechnology); rabbit anti-HA, 1:50 (Cell Signaling Technologies); rabbit anti-Vgll4 1:50 (Genescript, custom made). Primary antibody was washed 2-3 times with 0.1% PBST. Next, cells were incubated for 1 hour at room temperature with secondary antibodies raised in donkey and conjugated to Alexa fluorescent probes. All secondary antibodies were used at a 1:300 dilution. Cell nuclei were stained using DAPI at 1 μ g/ml.

3.8.11. Vgll4 custom-made antibody

An anti-human Vgll4 antibody was produced by Genescript. Rabbits were immunized with the following peptide: TANGDCRRDPRERSRSPIER. Antibody was validated by co-localization of HA staining and Vgll4 staining in fibroblasts transduced with Vgll4 lentivirus.

3.8.12. Luminescence assays for caspase activity

Cells were treated as described for the Annexin V/PI analysis. 15 thousand cells were collected for Caspase activity analysis. Caspase-Glo Assays (Promega) for Caspase 3/7 and Caspase 9 were used following manufacturer instructions. Caspase activity was measured using a luminometer optimized for measuring luciferase activity.

3.9 Note regarding author contributions

Dr. Julie Sneddon performed the analysis of microarray data with SAM to identify a list of significantly differentially expressed genes in Vgll4-hES cells.

Dr. José Rivera-Feliciano was instrumental for the design and interpretation of the experiments to determine an interaction between Vgll4 and Rho/Rock signaling.

References

- [1] H.-H. M. Chen, Steven J.; Stewart, Alexandre F.R., "Vgl-4, a Novel Member of the Vestigial-like Family of Transcription Cofactors, Regulates (alpha one)-Adrenergic Activation of Gene Expression in Cardiac Myocytes," *Journal of Biological Chemistry*, vol. 279, pp. 30800-30806, July 16, 2004 2004.
- [2] T. Maeda, D. L. Chapman, and A. F. Stewart, "Mammalian vestigial-like 2, a cofactor of TEF-1 and MEF2 transcription factors that promotes skeletal muscle differentiation," *J Biol Chem*, vol. 277, pp. 48889-98, Dec 13 2002.
- [3] P. Vaudin, R. Delanoue, I. Davidson, J. Silber, and A. Zider, "TONDU (TDU), a novel human protein related to the product of vestigial (vg) gene of *Drosophila melanogaster* interacts with vertebrate TEF factors and substitutes for Vg function in wing formation," *Development*, vol. 126, pp. 4807-16, Nov 1999.
- [4] K. J. Kaneko and M. L. DePamphilis, "Regulation of gene expression at the beginning of mammalian development and the TEAD family of transcription factors," *Dev Genet*, vol. 22, pp. 43-55, 1998.
- [5] G. Halder and S. B. Carroll, "Binding of the Vestigial co-factor switches the DNA-target selectivity of the Scalloped selector protein," *Development*, vol. 128, pp. 3295-305, Sep 2001.
- [6] R. Delanoue, K. Legent, N. Godefroy, D. Flagiello, A. Dutriaux, P. Vaudin, J. L. Becker, and J. Silber, "The *Drosophila* wing differentiation factor vestigial-scalloped is required for cell proliferation and cell survival at the dorso-ventral boundary of the wing imaginal disc," *Cell Death Differ*, vol. 11, pp. 110-22, Jan 2004.
- [7] S. Bray, "*Drosophila* development: Scalloped and Vestigial take wing," *Curr Biol*, vol. 9, pp. R245-7, Apr 8 1999.
- [8] M. Ohgushi, M. Matsumura, M. Eiraku, K. Murakami, T. Aramaki, A. Nishiyama, K. Muguruma, T. Nakano, H. Suga, M. Ueno, T. Ishizaki, H. Suemori, S. Narumiya, H. Niwa, and Y. Sasai, "Molecular pathway and cell state responsible for

- dissociation-induced apoptosis in human pluripotent stem cells," *Cell Stem Cell*, vol. 7, pp. 225-39, Aug 6 2010.
- [9] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J. B. Takahashi, S. Nishikawa, K. Muguruma, and Y. Sasai, "A ROCK inhibitor permits survival of dissociated human embryonic stem cells," *Nat Biotechnol*, vol. 25, pp. 681-6, Jun 2007.
 - [10] M. S. Samuel and M. F. Olson, "Dying alone: a tale of rho," *Cell Stem Cell*, vol. 7, pp. 135-6, Aug 6.
 - [11] M. Ohgushi and Y. Sasai, "Lonely death dance of human pluripotent stem cells: ROCKing between metastable cell states," *Trends in Cell Biology*, vol. 21, pp. 274-282, 2011.
 - [12] G. Chen, Z. Hou, D. R. Gulbranson, and J. A. Thomson, "Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells," *Cell Stem Cell*, vol. 7, pp. 240-8, Aug 6.
 - [13] V. G. Tusher, R. Tibshirani, and G. Chu, "Significance analysis of microarrays applied to the ionizing radiation response," *Proc Natl Acad Sci U S A*, vol. 98, pp. 5116-21, Apr 24 2001.
 - [14] G. Chen, Z. Hou, D. R. Gulbranson, and J. A. Thomson, "Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells," *Cell Stem Cell*, vol. 7, pp. 240-8, Aug 6 2010.
 - [15] Y. Onishi, Y. Azuma, Y. Sato, Y. Mizuno, T. Tadakuma, and H. Kizaki, "Topoisomerase inhibitors induce apoptosis in thymocytes," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1175, pp. 147-154, 1993.
 - [16] N. Droin, L. Dubrez, B. Eymin, C. Renvoize, J. Breard, M. T. Dimanche-Boitrel, and E. Solary, "Upregulation of CASP genes in human tumor cells undergoing etoposide-induced apoptosis," *Oncogene*, vol. 16, pp. 2885-94, Jun 4 1998.
 - [17] K. C. Chow and W. E. Ross, "Topoisomerase-specific drug sensitivity in relation to cell cycle progression," *Molecular and Cellular Biology*, vol. 7, pp. 3119-3123, September 1, 1987 1987.
 - [18] S. M. Van den Eijnde, L. Boshart, C. P. Reutelingsperger, C. I. De Zeeuw, and C. Vermeij-Keers, "Phosphatidylserine plasma membrane asymmetry in vivo: a pancellular phenomenon which alters during apoptosis," *Cell Death Differ*, vol. 4, pp. 311-6, May 1997.
 - [19] E. Ulukaya, C. Acilan, and Y. Yilmaz, "Apoptosis: why and how does it occur in biology?," *Cell Biochemistry and Function*, vol. 29, pp. 468-480.
 - [20] M. O. Hengartner, "The biochemistry of apoptosis," *Nature*, vol. 407, pp. 770-776, 2000.
 - [21] Y. Fuchs and H. Steller, "Programmed Cell Death in Animal Development and Disease," *Cell*, vol. 147, pp. 742-758.

Chapter 4

Elucidating the role of Vgll4 in human embryonic stem cells

4.1 Introduction

The function of Vgll4 remains largely unknown in mammalian cells. To characterize the mechanism of action of Vgll4, we explored several avenues in addition to a role in regulating apoptosis, as covered in Chapter 3. Ultimately, we chose to concentrate on characterizing the role of Vgll4 in survival of hES cells, however, several interesting leads explained in this chapter suggest that the role of Vgll4 is likely to be complex and include several pathways and modes of action.

4.2 A role for Vgll4 in the pluripotency network

4.2.1. Rationale

Vgll4 was identified in a gain-of-function genetic screen for genes that could maintain pluripotent cell identity under inhibition of TGF β /Activin/Nodal and FGF signaling –two pathways important for the maintenance of the hES cell state. Therefore, we tested the possibility of Vgll4 as a novel member of the pluripotency and self-renewal network in hES cells.

4.2.2. Results

A. Timecourse of pluripotency marker expression

We began by exploring whether Vgll4 could overcome long-term TGF β inhibition with SB-431542 in hES cells. To this end, we quantified the expression of pluripotency markers Tra1-60 and SSEA-3 in cells treated with DMSO (vehicle control) or 10 μ M SB-431542. We compared Vgll4-hES cells to WT hES cells as well as hES cells transduced with Nanog or GFP (**Figure 4-1**). Vgll4 was able to maintain higher proportions of pluripotent cells compared to WT and GFP. However, it was not sufficient to completely protect from differentiation, as in the case of Nanog.

This result could have several interpretations: 1) Vgll4 protects against differentiation but cannot fully halt it. Therefore, this results in a delay in differentiation, 2) high expression levels of Vgll4 for extended periods of time are deleterious to the pluripotent state. However, this second possibility is disproved by the

ability to maintain Vgll4-hES cells in self-renewing conditions for over 15 passages without loss of hES cell characteristics.

To test if Vgll4 was causing a delay in differentiation, we studied the dynamics of changes to colony morphology in cells treated with the combination of inhibitors for TGF β and FGF (TF). We observed a noticeable difference in the loss of hES cell colony morphology between Vgll4-hES cells and WT hES cells (**Figure 4-2**). At 5 days of treatment with TF, Vgll4-hES cells maintained the characteristic hES cell colony morphology with bright edges. By this timepoint, the majority of WT hES cells had lost colony morphology and differentiated. We remind the reader that these were the conditions used for the secondary verification assay of candidate genes (Chapter 2). Interestingly, in that assay we looked for candidates after 5 days of TF treatment and found Vgll4. Therefore, our results confirmed Vgll4 as a gene that can maintain colony morphology and pluripotency marker expression after 5 days of TF treatment. However, this ability of Vgll4 to maintain hES cell morphology was lost after 2 additional days of treatment. By Day 7 of treatment with TF, colony morphology had also been lost in most of the Vgll4 population.

We wondered if Vgll4 could also delay the loss of pluripotency markers under other differentiation conditions. Embryoid bodies formed from aggregates of ES cells recapitulate many events in early embryonic development [1]. For this reason, we aggregated cells to form embryoid bodies and assayed for changes in the mRNA levels of pluripotency markers Oct4 and Nanog for a period of 15 days.

EBs overexpressing Vgll4 were able to maintain levels of Oct4 and Nanog similar to those found in undifferentiated hES cells for approximately 2 days later into

differentiation than EBs expressing GFP or WT EBs. However the expression of these pluripotency markers was downregulated after this timepoint. Nanog EBs, in contrast, maintained high levels of Oct4 and Nanog throughout the entire timecourse (**Figure 4-3**).

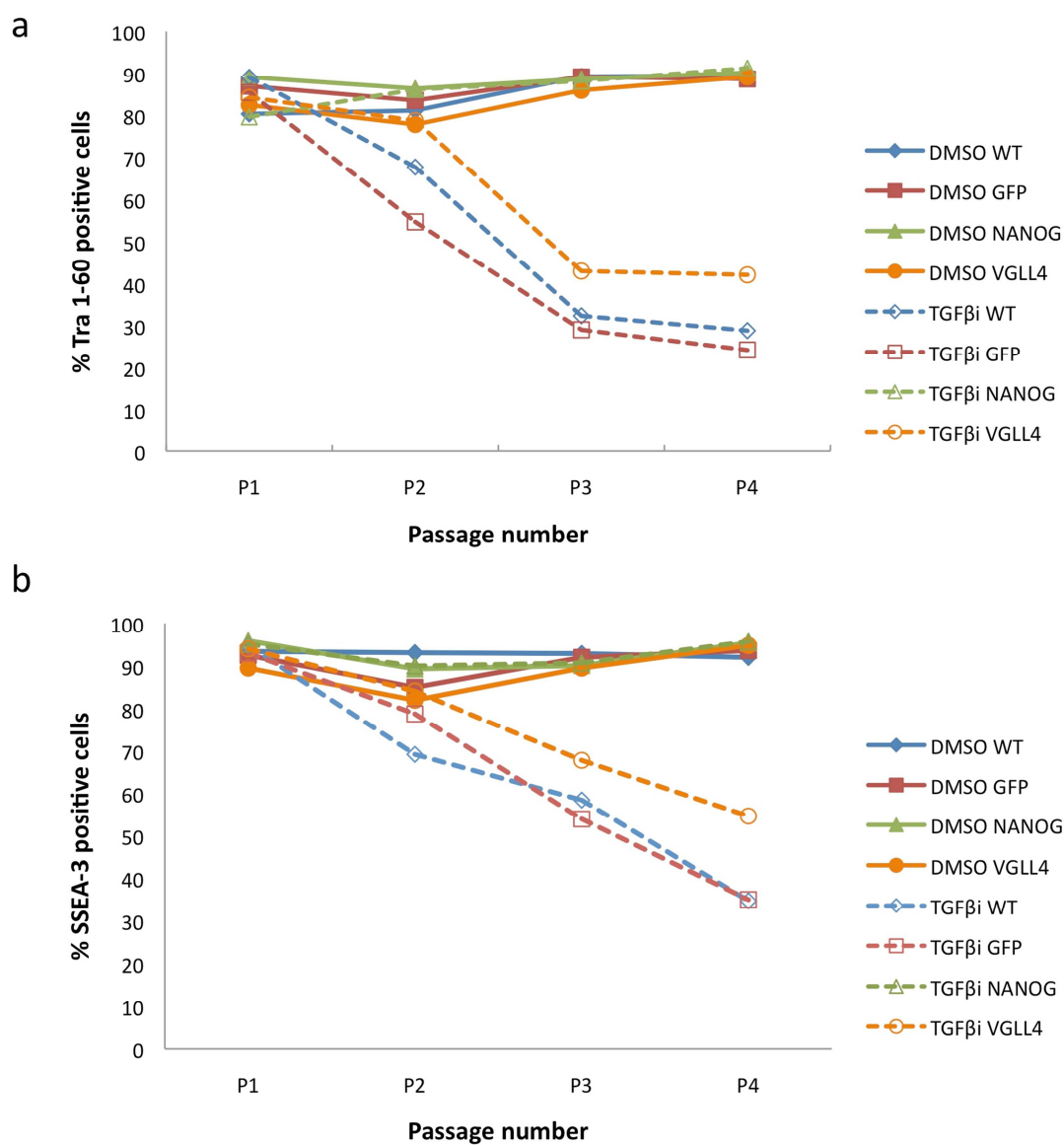


Figure 4-1. Vgll4 delays the loss of pluripotency markers in response to long-term treatment with SB-431542. Human ES cells were treated with SB-431542 (TGFβi) at 10μM for the number of passages indicated and assayed for the expression of pluripotency markers by flow cytometry. a) Expression of Tra1-60 over time. b) Expression of SSEA-3 over time. These results represent one experiment.

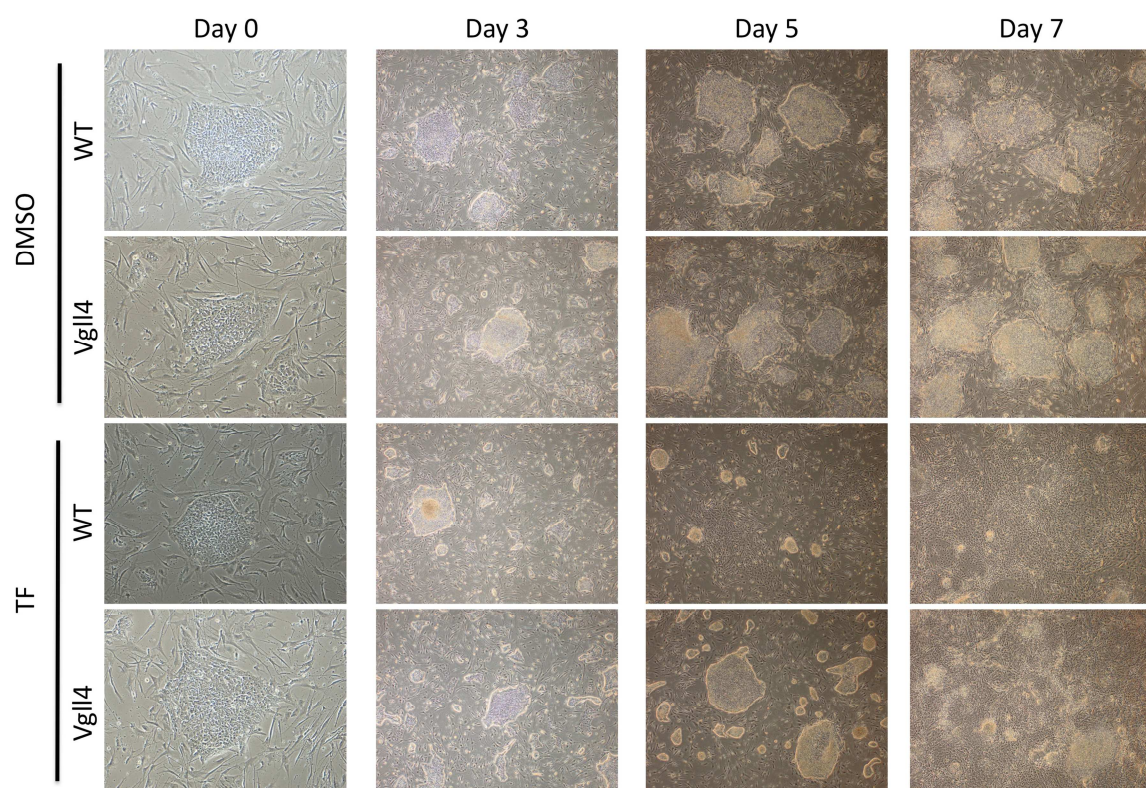


Figure 4-2. Vgll4 delays loss of colony morphology upon inhibition of TGF β and FGF signaling. Cells were treated with 10 μ M SB-431542 and 20 μ M SU-5402 (TF) for 7 days. Changes in colony morphology were monitored for Vgll4-hES cells and WT hES cells by light microscopy.

Finally, we tested whether Vgll4 would have a protective effect against differentiation in the presence of established differentiation protocols, for instance those used for directed differentiation of hES cells into definitive endoderm or mesoderm. When hES cells were differentiated into endoderm or cardiac progenitors, Oct4-positive cells were almost completely lost in all cell lines, including Nanog (**Figure 4-4**). Additionally, there was no difference in the induction of endoderm markers Sox17 and FoxA2 in the presence of Nanog, Vgll4, or GFP overexpression (**Figure 4-5**). This suggests that certain treatments override the ability to maintain pluripotency markers for extended periods of time.

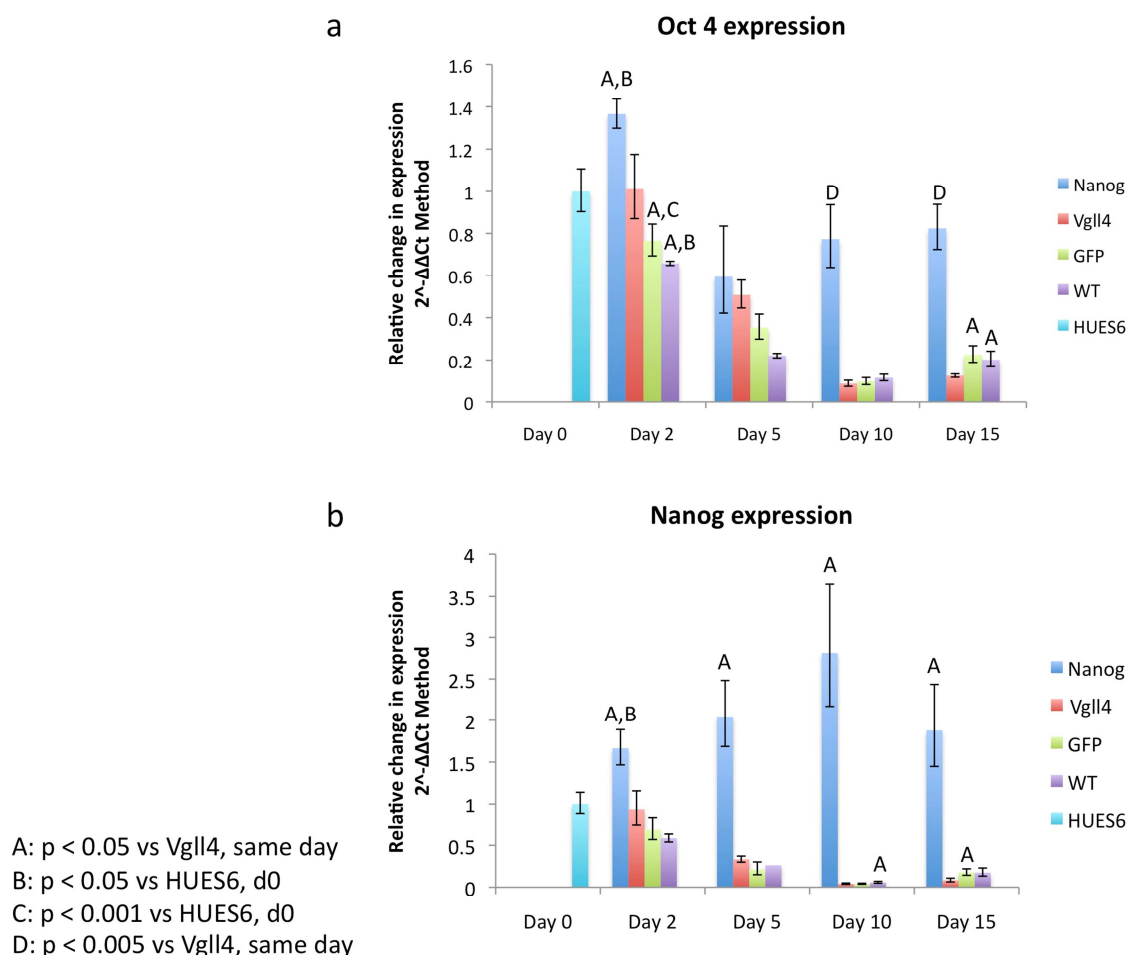


Figure 4-3. Vgll4 delays the loss of pluripotency markers during embryoid body differentiation. Embryoid bodies were formed from WT hES cells or hES cells transduced with Nanog, Vgll4, or GFP. mRNA levels of a) Oct4 and b) Nanog were assessed by real time PCR throughout a timecourse of 15 days. Levels of expression are relative to a GAPDH control. The blue bar represents endogenous expression of the gene in wildtype hES cells. P-values were calculated from Ct values using an unpaired Student's T-test.

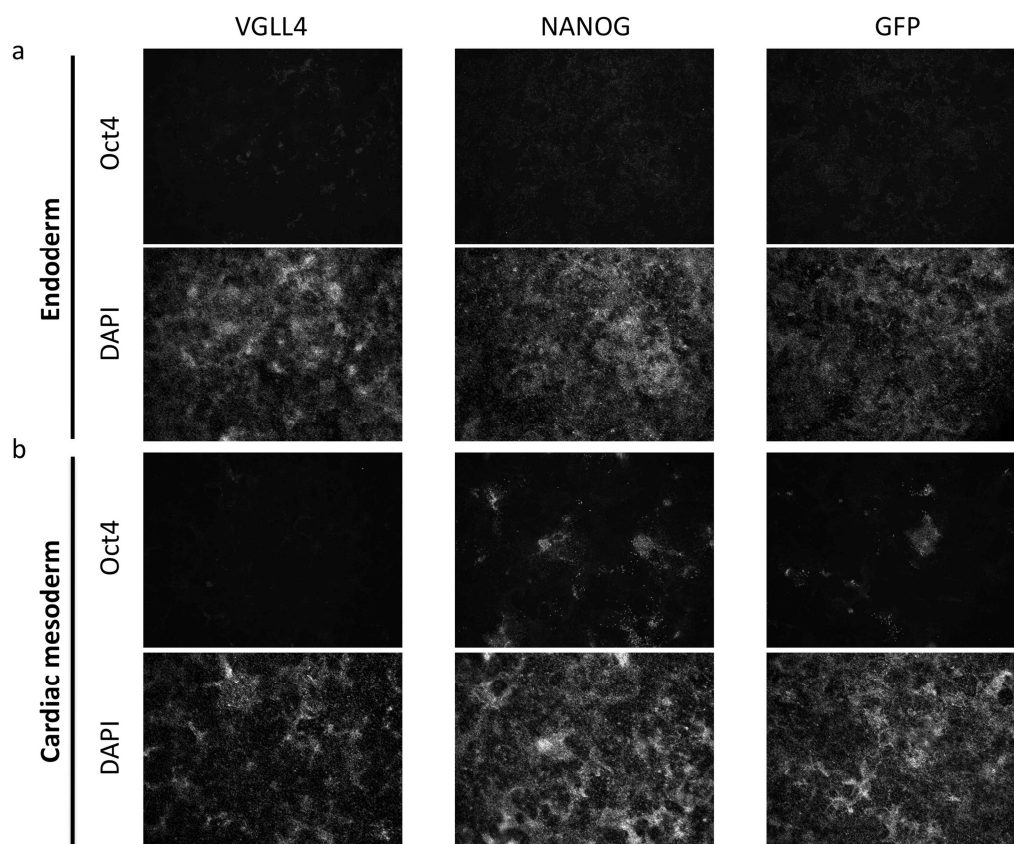


Figure 4-4. Overexpression of Nanog or Vgll4 is not sufficient to prevent the loss of Oct4 expression under directed differentiation protocols. Human ES cells overexpressing Nanog, Vgll4 or GFP were subjected to differentiation into a) endoderm or b) cardiac mesoderm progenitors. Oct4 expression was assessed at the end of the differentiation protocols.

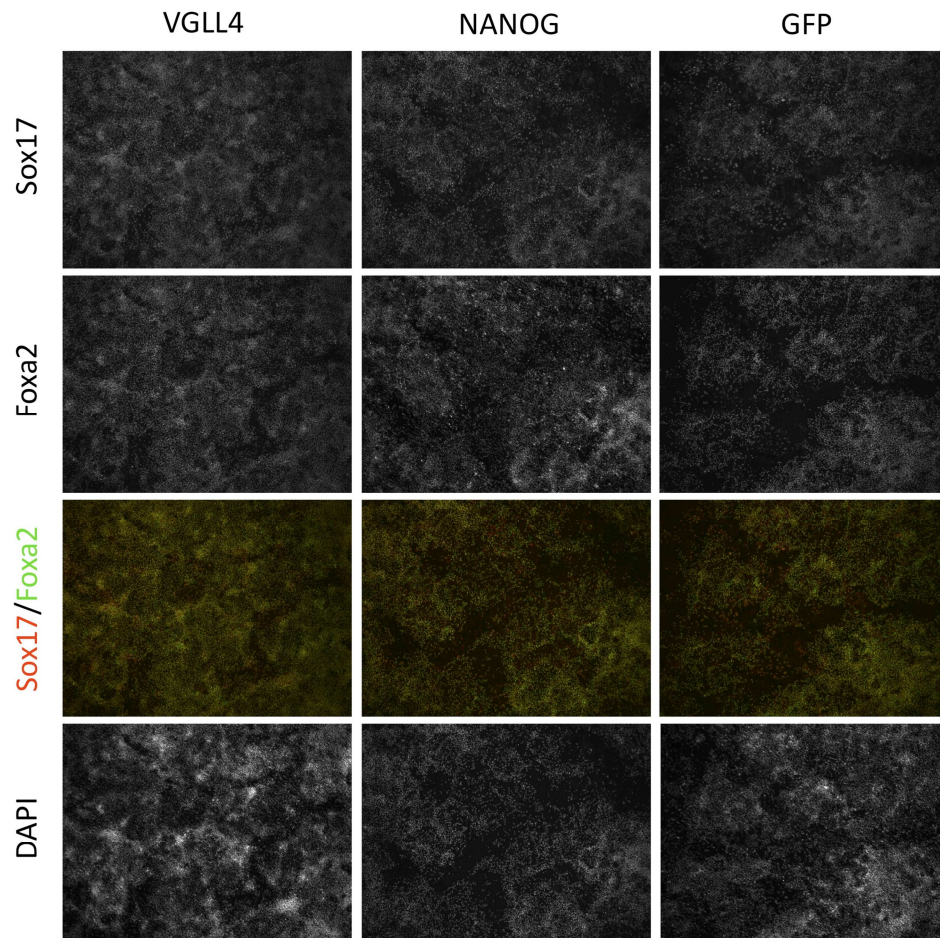


Figure 4-5. Human ES cells overexpressing Vgll4, Nanog, or GFP give rise to endoderm progenitors at similar efficiencies. Human ES cell lines were treated to induce differentiation into endoderm progenitors. The efficiency of differentiation was assessed by staining for Sox17 and FoxA2.

Our observations in response to treatment with inhibitors and EB formation, suggest that Vgll4 might have a protective effect against the loss of pluripotency markers, but our observations also show that some differentiation conditions might override overexpression of Vgll4 and even Nanog. Next, we also wanted to assess whether Vgll4 affected pluripotency, the ability of cells to differentiate into derivatives of the three germ layers. To this end, we examined whether hES cells overexpressing Nanog, Vgll4, or GFP would be able to form teratomas with cells from all germ layers. 3×10^6 to 1×10^7 hES cells overexpressing Nanog, GFP or Vgll4 were injected under the kidney capsule of SCID-Beige mice. Teratomas were isolated, embedded in paraffin, and sectioned after 40 days. We did not observe a difference in the frequency of teratoma formation between the different lines, but we noticed a difference in the size of the teratomas. Teratomas from Vgll4-hES cells were 2-3 times larger than those from Nanog or GFP hES cells. In all cases, we observed derivatives from the three germ layers (**Figure 4-6**). We attempted to detect the HA tag by immunofluorescence and expression of GFP but were unsuccessful in our attempts to optimize our staining protocols. Therefore we were not able to assess whether lentiviral expression was silenced throughout this process. Our observations allow us to conclude that overexpression of these genes at the hES cell stage does not interfere with multilineage differentiation.

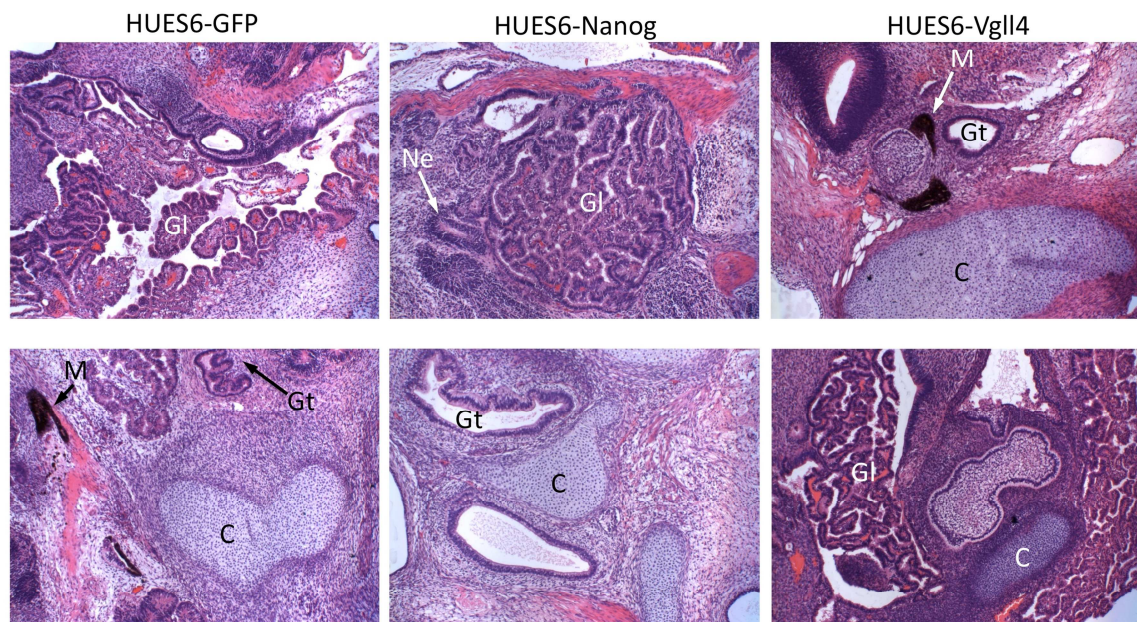


Figure 4-6. Nanog, Vgll4 or GFP overexpression are permissive to differentiation into the three germ layers by teratoma formation. Human ES cells overexpressing Nanog, Vgll4, or GFP were injected subcutaneously into immunocompromised mice. Derivatives from the three germ layers were identified by hematoxylin/eosin staining. Gl: glandular epithelium (endoderm), Gt: gut-like epithelium (endoderm), Ne: neural epithelium (ectoderm), M: melanocytes (ectoderm), C: cartilage (mesoderm).

B. *Vgll4* and the master pluripotency transcription factors

Our results showed that overexpression of *Vgll4* seemed able to delay the downregulation of pluripotency markers. Therefore, we tested whether downregulating *Vgll4* had an effect on the expression of pluripotency factors Oct4 and Nanog. We made use of V78, the shRNA against *Vgll4* introduced in Chapter 3. V78 achieves a 60% reduction in the levels of *Vgll4* by real-time PCR. In cells where the level of *Vgll4* has been reduced, the levels of Nanog were decreased by 40%. In addition, the level of Oct4 also decreased by 40% in some cases (**Figure 4-7**).

Conversely, to test whether changes in the levels of pluripotency genes would affect *Vgll4* expression, we tested several shRNAs against Nanog, referred to as N52-1, N52-2, N53-1, N53-2, N55-1, and N55-2. These hairpins achieved a reduction in the level of Nanog mRNA of 30-80%. In most cases, a reduction in Nanog levels correlated

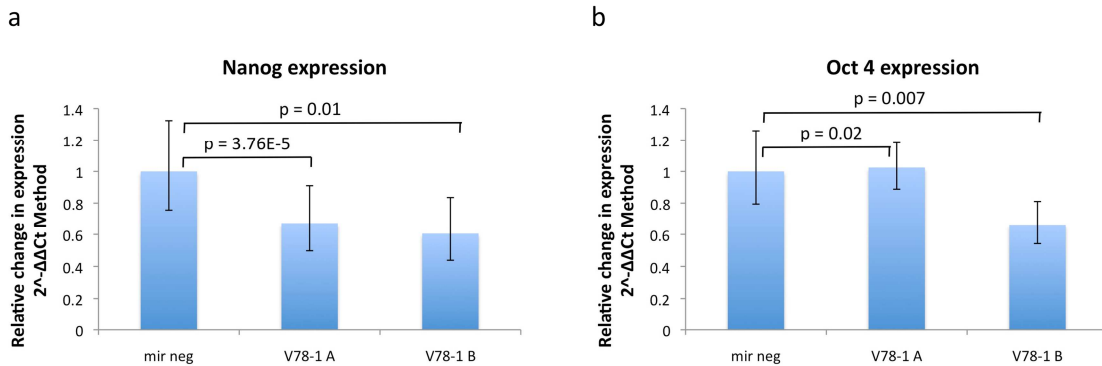


Figure 4-7. Expression levels of pluripotency factors respond to *Vgll4* downregulation.

The levels of *Vgll4* were reduced by approximately 60% using V78, a shRNA against *Vgll4*. Nanog and Oct4 expression were assessed by real-time PCR. a) Nanog mRNA levels in cells treated with a scrambled control (mir neg) or V78. b) Oct4 mRNA levels in cells treated with a scrambled control (mir neg) or V78. A and B represent two independent viral production rounds and transductions. P-values were calculated from Ct values using an unpaired Student's T-test.

with an increase in the levels of Vgll4. This could suggest the existence of a negative feedback loop where Nanog is induced by Vgll4 and Vgll4 is inhibited by Nanog (**Figure 4-8**). A similar inverse correlation was observed previously (Chapter 3, Figure 1) where Nanog was reduced by allowing cells to differentiate in EB formation, and levels of Vgll4 concomitantly increased. It is also possible that levels of Vgll4 increase simply due to differentiation. Therefore, distinguishing between a direct and indirect effect in the regulation of Vgll4 by pluripotency genes still remains open to investigation.

Interestingly, some indications in the literature suggest that the regulation of Vgll4 by pluripotency genes might be direct. For instance, the Vgll4 promoter region has been reported to be bound by Oct4, Sox2, and Nanog in mouse ES cells through ChIP-seq analysis [2]. Data from this study also shows that the Vgll4 promoter harbors dimethylation at lysine 79 in histone H3 (H3K79me2), an epigenetic mark associated with active expression. However, this study also reports that the Vgll4 promoter is bound by Tcf3, commonly but not always associated with repression [3]. Determining whether Vgll4 is expressed in mES cells would be helpful to resolve whether Vgll4 is positively or negatively regulated by the pluripotency genes in ES cells. This information would be informative towards establishing a model for the regulation of Vgll4 in self-renewal and differentiation.

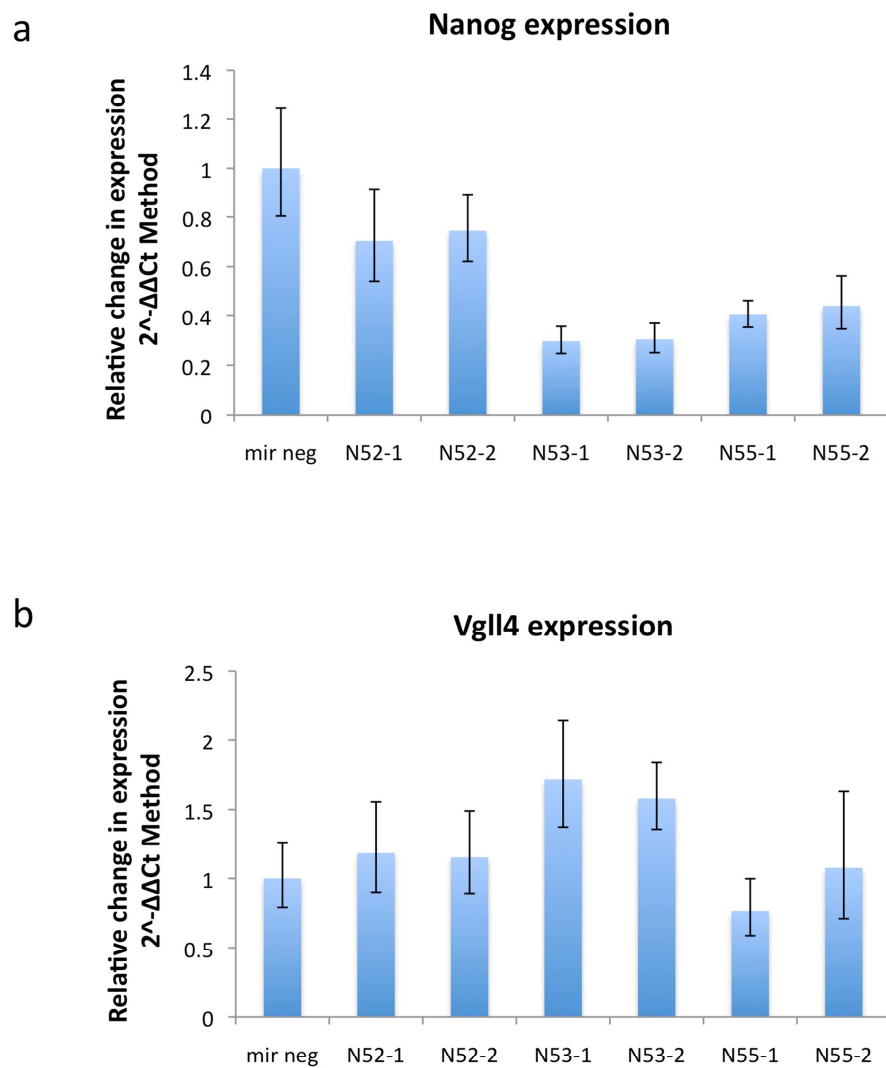


Figure 4-8. Downregulation of Nanog levels lead to an increase in Vgll4 expression. a) shRNAs (N52, N53, N55) against Nanog achieve 30-80% reduction in Nanog mRNA levels. b) Levels of Vgll4 were assessed in response to Nanog knockdown with the indicated shRNAs.

C. Reprogramming into iPS cells

Reprogramming of adult cells into the embryonic stem cell state can be achieved by the overexpression of some of the core pluripotency transcription factors, including Oct4, Sox2, and Nanog [4-8]. Many advances have been made for improving the efficiency of this process [9].

Our results have shown that Vgll4 regulates the survival of pluripotent cells and may delay differentiation. In addition, Vgll4 might also reinforce Nanog expression. For these reasons, we were interested in determining whether Vgll4 had a role in reprogramming. We tested whether Vgll4 would affect the efficiency of reprogramming together with the reprogramming factors Oct4, Sox2, and Klf4. Human fibroblasts from narcoleptic patients (obtained from Dr. Florian Merkle, Eggen Lab, Harvard University) were transduced with retroviruses for the human genes Oct4, Sox2, and Klf4. In addition GFP or Vgll4 lentivirus was added. In two independent attempts, we observed that the efficiency of reprogramming was lowered upon Vgll4 overexpression. This result could have several interpretations. First, that Vgll4 acts as an inhibitor of reprogramming. This could result from inhibiting a pathway necessary for reprogramming or from activating pathways that stabilize the current cell state and make the necessary cell changes for reprogramming more difficult.

We certainly think that further study of this question is necessary. This experiment was mostly exploratory and several conditions should be optimized to get a strong conclusion. For instance, it would be ideal to repeat this experiment using WT human fibroblasts. Even though cells with multiple disease backgrounds have been reprogrammed, cells from narcoleptic fibroblasts could harbor important differences in

their reprogramming kinetics and mechanisms. Second, a careful tabulation of the number of resulting colonies is necessary to determine the effect to reprogramming efficiency. Finally, exploring the timing of *Vgll4* expression would be interesting to determine whether it becomes a permissive factor further down the reprogramming process.

4.2.3. Conclusions

We found Vgll4 in a screen designed to uncover genes important for hES cell maintenance. However, our results show that, unlike Nanog, Vgll4 overexpression is not sufficient to prevent the loss of pluripotency markers and differentiation upon extended exposure to differentiation inducers. Nanog has been shown to overcome differentiation-inducing conditions such as feeder-free growth, to maintain the pluripotent state in hES cells [10]. In our assays (long-term TGF β inhibition and EB formation) Nanog overexpression is able to maintain expression of pluripotency markers Tra 1-60, SSEA-3, and Oct4. One possible explanation is that in the TGF β timecourse, Nanog can overcome differentiation since it has been shown to be a downstream target of TGF β signaling in hESCs, thus counteracting the effect of the inhibition [11]. Additionally, it is possible that we detect Oct4 expression because its expression is regulated by Nanog as part of a feedforward loop [12]. It would be important to characterize the expression of differentiation markers to better assess the ability of Vgll4 to delay differentiation and the ability of Nanog to inhibit it.

Nanog overexpression was not sufficient to overcome treatment with endoderm or cardiac mesoderm-induction protocols. This would suggest that the use of direct inducers of differentiation is sufficient to destabilize the pluripotency transcriptional network despite overexpression of one of its components. This experiment also allows us to conclude that Vgll4 overexpression does not interfere with directed differentiation into these lineages. It would be interesting, however to more carefully study the timepoints at which these markers arise to determine if Vgll4 is delaying differentiation in this context.

Formation of teratomas with derivatives from the three germ layers were obtained for hES cells overexpressing, Nanog, GFP, or Vgll4. Interestingly, Vgll4 teratomas were approximately 2-3 times the size of Nanog and GFP teratomas (data not shown). Since we were unable to assess continued lentiviral expression, we cannot make conclusions about the effect of these genes on the differentiation into certain lineages, but we can conclude that overexpression of these genes does not irreversibly affect the pluripotency of hES cells. This point would have to be examined more closely by directly comparing the efficiency of differentiation into specific cell types, for example by directed differentiation. Nevertheless, this point is important if, for instance, Vgll4 were to be used as a method to expand the numbers of pluripotent stem cells by increasing survival.

Interestingly, downregulation of Vgll4 seems to have an effect on the expression levels of Nanog, and perhaps Oct4. The biological relevance of these changes remains to be characterized but it is suggestive of a role for Vgll4 in the stabilizing the pluripotent transcriptional network. Conversely, we detected increased levels of Vgll4 mRNA upon downregulating Nanog. One possible interpretation of this result is that Nanog and Vgll4 could regulate each other as part of a negative feedback loop. An alternative interpretation is that since Vgll4 increases upon differentiation, this represents an increase in a differentiated population. Also, the fact that N55 hairpins did not increase levels of Vgll4 despite considerable downregulation of Nanog presents a need for further investigation.

The fact that Vgll4 did not positively affect the efficiency of reprogramming was surprising given the roles we have uncovered for this gene in hES cells, including

survival, delay of differentiation and possible regulation of pluripotency genes. However, given the role of Vgll4 as a co-transcriptional regulator, and the widespread expression of TEAD factors, adding Vgll4 could be helping to stabilize the differentiated state. To further address this question, it would be interesting to test the response to addition of Vgll4 at different timepoints in the reprogramming process, especially later on. In addition, optimization of our reprogramming conditions, as explained above, should be helpful to draw a more definitive conclusion. It is interesting to point out that results from a recent study indicated that the TAZ co-transcriptional regulator is necessary for successful reprogramming [13]. As explained in Section 4 of this Chapter, if Vgll4 is acting as a negative regulator of the YAP and TAZ co-transcriptional regulators, the decrease in reprogramming efficiency that we observed would be consistent with the findings of that study.

4.3 A role for Vgll4 in the regulation of proliferation

4.3.1. *Rationale*

Overexpression of Vgll4 increased the rate of population doubling in a variety of pluripotent lines (please refer to Chapter 3, Section 3). This result could be explained by an increased rate of replication, a decrease in the rate of cell death, or a combination of these two mechanisms. For this purpose, we tested whether Vgll4 was affecting cell cycle dynamics in hES cells.

4.3.2. *Results*

To assess whether Vgll4 was affecting cell cycle dynamics in hES cells, we began by looking at the distribution of cells in different phases of the cell cycle. An assessment of cell cycle distribution in different transduced lines was achieved by measuring DNA content by propidium iodide (PI) staining and flow cytometry. In most cases tested using different pluripotent lines, Vgll4 did not seem to affect the distribution of cells in the cell cycle. However, we found it technically challenging to replicate this assay consistently in order to build strong statistics supporting our conclusion (**Figure 4-9**).

To further explore whether Vgll4 was affecting cell cycle dynamics, we have begun to use markers for different phases of the cell cycle as a complementary way to get an accurate representation of cell cycle distribution. We used phospho-histone H3 (pH3) to detect the proportion of mitotic cells under self-renewing conditions 5-6 days after plating. We found a similar proportion of mitotic cells in our different cell lines (**Figure**

4-10), suggesting that Vgl4 is not acting to increase the proportion of cells undergoing mitosis.

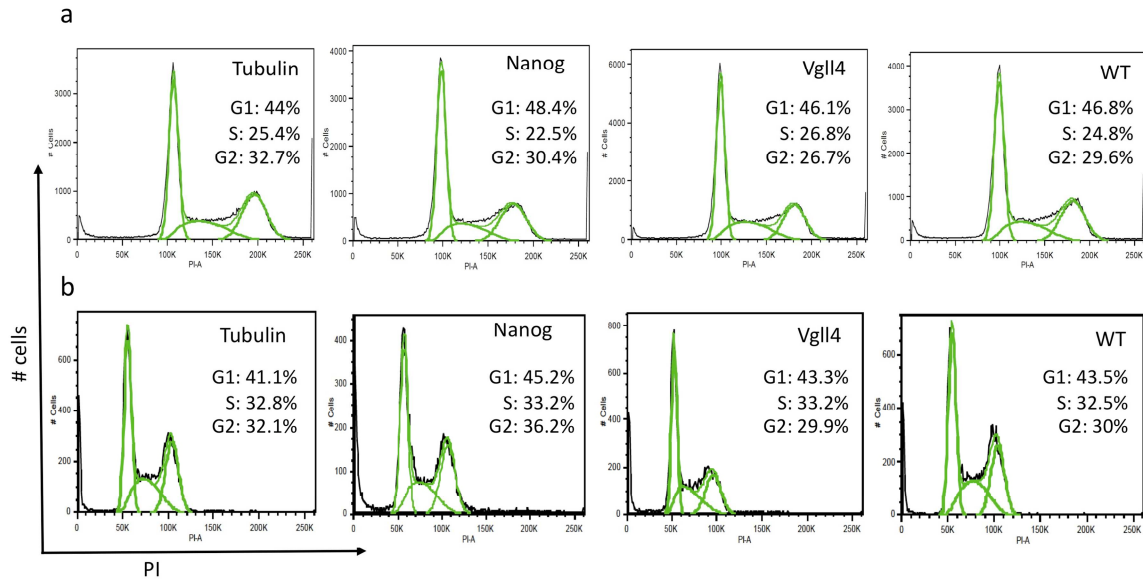


Figure 4-9. Distribution of cell populations in the cell cycle. Two representative experiments (a and b) are shown. Distribution of cells in the cell cycle was assessed by DNA quantification using PI staining. The Dean-Jett-Fox model was used for calculating the area under the curve. The reason behind G1, S, G2 percentages adding up to more than 100% is that the modeling algorithms contain some approximations and are rounded numbers.

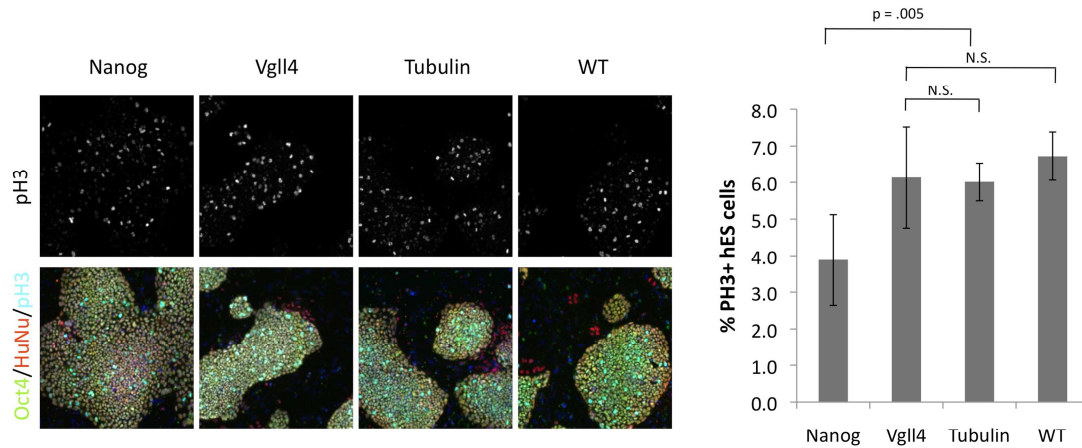


Figure 4-10. Proportion of mitotic cells in self-renewing hES cell lines. Human ES cell lines were fixed 5-6 days after plating and stained with Oct4, Human Nuclear Antigen (HuNu), phospho-histone H3 (pH3) and DAPI. Panels to the left show representative images of mitotic cells and quantification is provided to the right. Significance was calculated using an unpaired Student's T-test. N.S. = not significant.

4.3.3. Conclusions

We began to explore whether Vgll4 has a role in regulating cell cycle dynamics of hES cells that could contribute to the 2-3 fold higher population doubling rate observed in multiple cell lines. Even though our results from examining the distribution of cells in different phases of the cell cycle by assessing DNA content did not indicate a substantial difference, our approach was technically challenging to reproduce consistently and many experiments could not be analyzed because of too much noise in the sample. We think that these problems could be due to cell loss during sample preparation, cell aggregation during analysis, and inherent noise in PI labeling. Additionally, variations in the distribution of cells between experiments provide a challenge for statistical analysis. We were careful to use cells prepared exactly under the same conditions. Therefore, we think that these variations are not biologically significant and likely have to do with variables such as small differences in the density of cell culture upon harvesting. Finally, the Dean-Jett-Fox model for calculating the area under the curve gives a more accurate estimation of cell cycle distribution compared to regular gating. Nevertheless, the model requires a near perfect cell cycle outline to fit clearly, and if this is not the case, it rejects the data. An alternative is to do some manual adjustments of the model, but this does not get rid of the problem of noise in the samples.

To overcome these challenges, we have begun to assess specific markers for cell cycle phases by flow cytometry and immunocytochemistry. This approach has a better signal-to-noise ratio than PI labeling and can provide us with more accurate information than the curve-fit algorithm. A caveat of our approaches so far, however, is that they are only providing us a snapshot of the cell cycle distribution at a particular

timepoint. It is possible that the changes will be more noticeable at early timepoints after plating. Therefore, we are currently continuing to assess differences in markers of all phases of the cell cycle and exploring early and late timepoints after plating to detect any differences in the cell cycle distribution of our cell populations. Understanding whether *Vgll4* alters cell cycle dynamics is instrumental to devise approaches to use this gene for the expansion of hES cell populations.

4.4 A role for Vgll4 in the Hippo pathway

4.4.1. *Rationale*

The Hippo pathway regulates, proliferation, apoptosis, differentiation, and organ size in flies and mammals. Components of this pathway in mammals are closely conserved to their counterparts in *Drosophila* [14]. Briefly, Mst1/2 kinase lies at the core of the pathway. Mechanisms for its activation are still being uncovered, but activated Mst1/2 phosphorylates and activates Lats1/2. Lats1/2, in turn phosphorylates and inhibits the co-transcriptional regulators Yap and Taz. Thus, when the Hippo pathway is inactive, Yap and Taz can translocate into the nucleus and interact with TEAD to promote gene expression [14-16]. Upstream activators of the pathway are still being discovered, but there is increasing evidence of a relation to cell polarity and adhesion [17, 18].

Besides its roles in tissue homeostasis, the Hippo pathway has been shown to play an important role in the initial cell fate decisions in the mouse embryo. Differential localization of Yap to the outside cells of the early embryo promotes activation of trophectoderm versus inner cell mass genes [18]. Interestingly, the Hippo pathway has also been shown to have a role in pluripotency. Yap has been shown to be important for mES cell self-renewal [19] and TAZ was shown to be necessary for iPS reprogramming [13]. Given its interactions with TEADs [20], we wondered if Vgll4 could be acting as a regulator of the Hippo pathway in hES cells.

A previous study by Chen et al., identified Vgll4 as a negative regulator of TEAD-mediated expression in murine cardiomyocytes [20]. Therefore, we were interested in

testing whether Vgll4 would be a negative regulator for TEAD-mediated activity. Our approach to this question was two-fold: First, we looked for downregulated TEAD target genes in our microarray data of WT hES cells versus hES cells overexpressing Vgll4. Second, we used a reporter cell line for TEAD activity to assess whether TEAD-mediated transcription was suppressed in the presence of Vgll4.

4.4.2. Results

To test whether Vgll4 was a novel regulator of the Hippo pathway, we began by searching for evidence of downregulation of TEAD target genes in our microarray data from WT and Vgll4-overexpressing hES cells. CTGF (connective tissue growth factor) and CYR61 (cysteine-rich, angiogenic inducer, 61) have been shown to be direct target genes of the Hippo pathway in mammalian cells [14, 21, 22]. Both of these genes were significantly downregulated in hES cells in response to Vgll4 overexpression.

Next, in collaboration with Fernando Camargo (Harvard University), we assessed the impact of Vgll4 overexpression on the activation of TEAD-mediated transcription by using a reporter plasmid for TEAD activity. This reporter, generated in the Camargo Lab, contains TEAD-binding sequences upstream of a luciferase gene. Cells were transfected with the reporter plasmid in addition to Vgll4, GFP (negative control) or Yap S127A (a constitutively active version of Yap, positive control). From this experiment, Vgll4 seems to have an inhibitory role in TEAD-mediated transcription (**Figure 4-11**). This agrees a previous report in the literature that suggests that Vgll4 inhibits transcription activation in cardiomyocytes [20]. To our surprise, Vgll4 was even able to decrease the activation caused by constitutively active Yap. To ensure that this was not an artifact caused by halving the effective concentration of Yap S127A, we added a control where Yap S127A and GFP were co-transfected. This co-transfection did not alter the signal compared to S127A alone, indicating an effect for Vgll4 in decreasing TEAD-mediated transcription.

To test the relevance of this result, we would ideally test it in hES cells to ensure the absence of cell-context specificity effects. Since decided to focus our efforts to

exploring the role of Vgll4 in hES cell survival, these experiments remain to be done in the future.

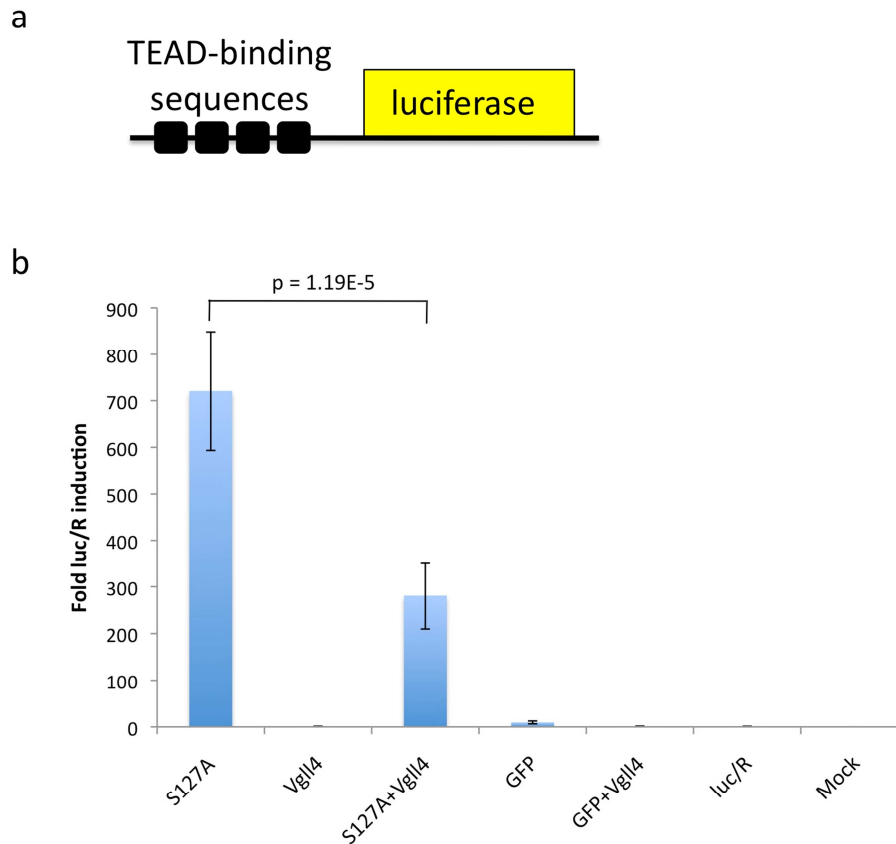


Figure 4-11. Vgll4 inhibits TEAD-mediated transcription in a reporter assay. 293T cells carried the reporter plasmid outlined in a). This reporter contains TEAD binding sequences in front of a luciferase gene. b) Cells were transfected the reporter plasmid and Renilla as a transfection control in addition to the indicated genes. Activation of the reporter was assessed as a function of luciferase/Renilla luminescence. Mock = treated only with transfection reagents, luc/R = transfected only with reporter plasmid and Renilla control, S127A = constitutively active Yap. Results represent 3 biological replicates in duplicates. P-value was obtained with an unpaired Student's T-test.

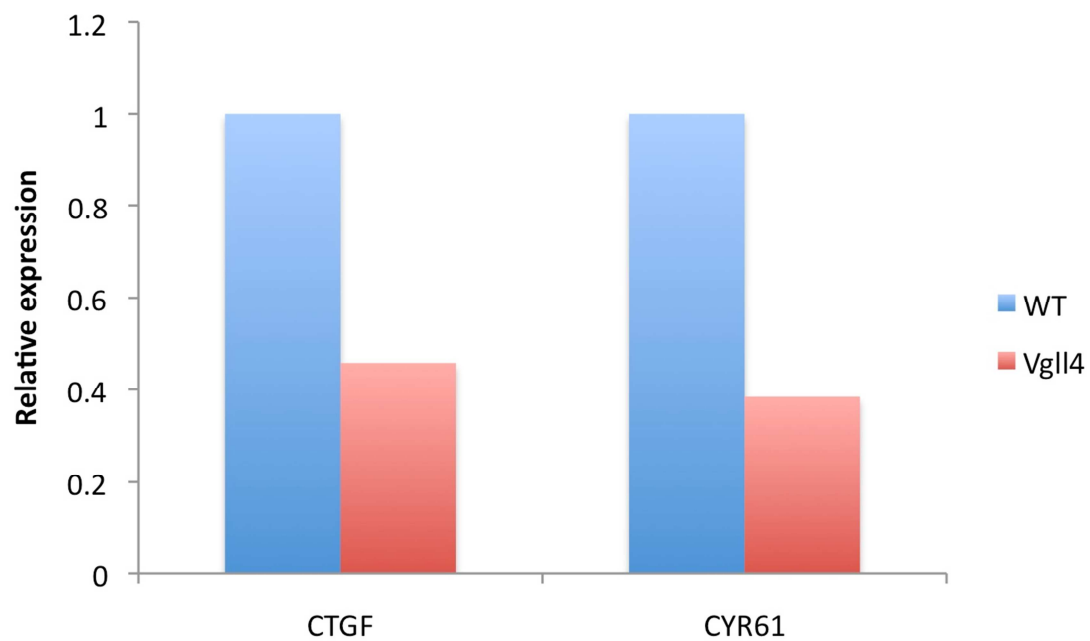


Figure 4-12. Vgll4 downregulates Hippo pathway target genes in hES cells. Relative expression changes for pro-apoptotic genes in Vgll4 relative to WT hES cells were obtained by microarray analysis. The q-values for these changes in expression are: CTGF q = 0%; CYR61 q = 0%. Q-values were obtained by analysis with SAM.

4.4.3. Conclusions

The importance of the Hippo pathway for many cellular decisions is quickly becoming clear. We became interested in exploring a role for Vgll4 in this pathway because of its homology to members of the *Drosophila* signaling pathway, as well as its interaction with known effectors downstream of the Hippo pathway in mammalian cells. Our results suggest that Vgll4 may act as an inhibitor of transcriptional targets of TEAD transcription factors, consistent with a previous report. However, more work is necessary to fully understand and characterize a possible role for Vgll4 in this pathway.

Nevertheless, the prospect of a novel inhibitor of the pathway is promising. Vgll4 has been reported to be a negative regulator of TEAD-dependent gene expression in cardiomyocytes [20]. Given the roles reported for the Hippo pathway in mediating early cell fate decisions, self-renewal, and reprogramming, a negative regulator for this pathway would be interesting and important. TEAD target genes were downregulated upon Vgll4 overexpression in hES cells, suggesting that our observation in reporter cells might be relevant in the hES cell context. It is of note that this downregulation happened within the highest confidence interval and significance according to analysis with SAM.

There is, nevertheless, an alternative explanation to these observations. It is possible that Vgll4 is altering the DNA-binding selectivity of TEAD, in a way competing it away from its known YAP-mediated targets. In *Drosophila*, Vg has been reported to change the DNA specificity of Scalloped (Sd), the TEAD homolog [23] through a mechanism that is not understood. In flies, this target selectivity allows the activation of a different set of genes by Sd depending on whether it is bound by Yorkie (Yki, the

Yap/Taz homolog) or by Vg. More work is necessary to determine whether our observed decrease in TEAD-mediated activity is caused by an inhibition of Yap-TEAD complexes by Vgll4 or by switching the target selectivity of TEAD when bound to Vgll4.

The Hippo pathway was recently shown to have an important role in mediating one of the first cell fate decisions in the development of the mouse embryo: the trophoctoderm versus inner cell mass specification. In this study, the authors find Yap localized to the nucleus of cells on the outside of the embryo that then go on to become trophoctoderm. The authors propose that there might be a factor that interacts with TEAD and antagonizes Yap in inside cells to set up an inner cell mass program [18].

Additionally, there is increasing evidence for a role of the cytoskeleton and extracellular matrix cues as regulators of Hippo pathway signaling. A recent study suggests that Yap and Taz respond to ECM cues. In this context, Yap and Taz activity was dependent on Rho signaling [24], which has also been shown to promote the myosin hyperactivation leading to apoptosis in hES cells [25-27]. Of course, these studies were done with mammary epithelial cell lines and the relevance of these findings would have to be confirmed in hES cells, but it could suggest another interesting link with our findings from Chapter 3 and the possibility that Vgll4 and Rock are acting as part of the same pathway.

4.5 A role for Vgll4 in the regulation of TGF β signaling

4.5.1. Rationale

Vgll4 was originally identified in our screen as a gene whose expression allowed hES cells to maintain their pluripotent state in the presence of TGF β inhibitor. One obvious explanation for this phenotype would be that Vgll4 was acting downstream in this pathway to counteract the inhibition of signaling. For this reason, we investigated whether the overexpressed Vgll4 could be acting downstream of the TGF β receptor to restore activity of this signaling pathway, in the context of receptor inhibition by SB-431542.

4.5.2. Results

To detect TGF β signaling activity, we used a mink lung epithelial reporter cell line. This cell line, known as TMLC, stably contains a portion of the plasminogen activator inhibitor 1 (PAI-1) promoter fused to a luciferase reporter gene [28] and has been widely used in the TGF β -signaling field [29, 30]. We transduced these cells with GFP or Vgll4 and selected for successfully transduced cells with puromycin. These cells were then treated for 12 hours with the following treatments: vehicle control, TGF β (50 ng/ml), SB-431542 at 10 μ M, and TGF β +SB-431542. Following treatment, luciferase activity was measured (**Figure 4-13**).

Unexpectedly, Vgll4 appeared to inhibit activation of the PAI-1 promoter. This result was surprising since we expected Vgll4 to counteract the effect of TGF β inhibition in hES cells. Even though this experiment is informative, there are several caveats to the

interpretation of these results. First, it is necessary to rule out species- and cell-context specific effects. Especially given that *Vgll4* is a co-transcriptional regulator, its effects could be context-dependent. Second, this assay is based on a single promoter. To better assess whether this is a global effect on TGF β signaling, looking at a wider range of genes is necessary. This second point is especially important given that the molecules thought to be more important in hES cell pluripotency are Activin and Nodal [31-34]. Even though TGF β signaling uses many of the same signal transducers, the effect on downstream targets can vary slightly [35].

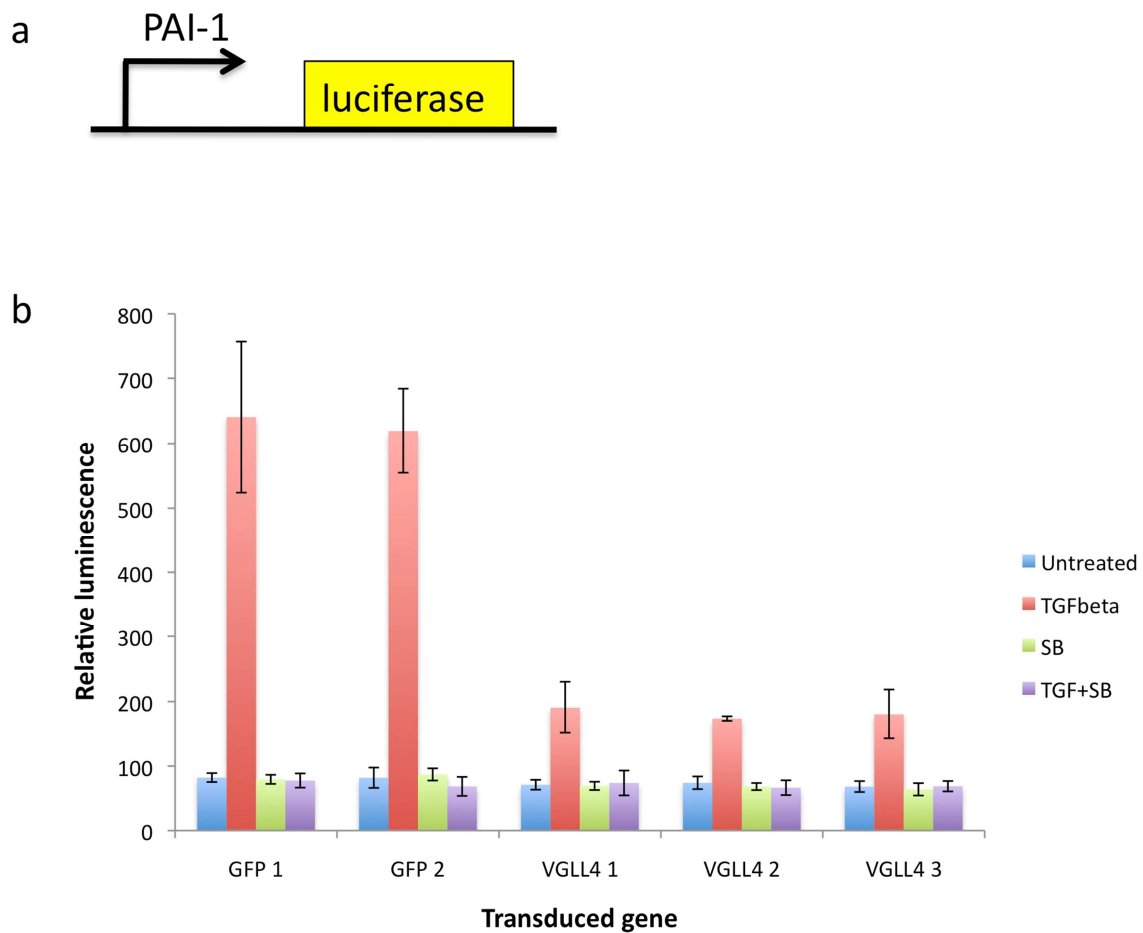


Figure 4-13. Vgll4 inhibits the activation of a gene target of TGF β signaling. a) TMLC reporter cells carry a portion of the plasminogen activator inhibitor 1 (PAI-1) driving expression of a luciferase gene. b) TMLC lines overexpressing GFP or Vgll4 were treated with the indicated treatments for 12 hours and luciferase activity was assessed.

4.5.3. Conclusions

This result is puzzling given that Vgll4 was found as a gene that could overcome TGF β inhibition as a means of inducing differentiation in hES cells. Unless Vgll4 stabilizes other nodes of the pluripotency network despite the inhibition of this pathway, it would be difficult to reconcile this result in the context of hES cell maintenance. Additionally, hES cells transduced with Vgll4 can be maintained and passaged for over 30 passages without loss of pluripotency markers. An alternative explanation is that Vgll4 acts as a transcriptional repressor and that when expressed at higher levels it quenches regulators needed to activate TGF β signaling.

An alternative, and perhaps simpler, explanation is that PAI-1 (also known as SERPINE1) has been reported to be a target for Yap/TEAD [36]. Interestingly, several studies have shown an interaction between Yap and Taz and SMAD shuttling and signaling [37, 38]. The specific nature and domains of interaction between Yap, Taz, and Smads are unknown. However, if the interaction occurs through a domain shared between Yap, Taz, and Vgll4, then the overexpression of Vgll4 could sequester Smad. There is currently no evidence to support a direct interaction between Vgll4 and Yap or Taz, although that could be an alternative explanation. Nevertheless, it is still difficult to reconcile this result with the observed phenotype of hES cell maintenance.

4.6 Concluding remarks

Overall, the activity of Vgll4 seems to be complex and to span more than one mode of action. In this chapter we explored four possible mechanisms for Vgll4 activity and found evidence to support a role in most of them. We are aware that these results need

to be extensively verified but we hope that they would serve as a foundation for further characterization of this gene in hES cells.

4.7 Methods used in this chapter

4.7.1. *hES cell culture*

HUES6 were plated on irradiated murine embryonic fibroblasts (MEFs) and grown in media containing KO-DMEM (Invitrogen), 10% Knockout Serum Replacement (KOSR, GIBCO), 10% human plasma fraction (Talecris), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (GIBCO), .055 mM β -Mercaptoethanol (GIBCO), and 10 ng/ml bFGF (Invitrogen). Cultures were passaged using 0.05% Trypsin-EDTA (GIBCO) at a ratio of 1:6-1:10 every 5-7 days.

Where indicated, SB-431542 was used at a concentration of 10 μ M, SU-5402 at 20 μ M. Both of these were resuspended in DMSO. DMSO vehicle controls were made with the equivalent volume of DMSO of the two chemicals combined. Retinoic Acid was used at a final concentration of 10 μ M.

MEFs were plated on plates coated with 0.1% gelatin. MEF media contained 1X DMEM (Mediatech), 10% FBS, 2mM L-glutamine (Invitrogen), and 0.1mM non-essential amino acids (GIBCO).

4.7.2. *Assessment of pluripotency markers by flow cytometry*

Cells transduced with Nanog, GFP, or untransduced (WT) were cultured in the presence of 10 μ M SB431542 (TGFbi) or with vehicle control (the equivalent amount of DMSO). Cells were passaged every 5-7 days. A fraction of cells was re-plated and the rest was used to analyze the level of pluripotency markers by FACS analysis. After washing the pellet with PBS, the cells were resuspended in a 1:100 dilution of Tra1-60

antibody (Millipore) and incubated on ice for 40 minutes. Cells were then washed and resuspended in a secondary antibody solution of APC-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch) at a concentration of 1:300. Cells were incubated for 20 minutes on ice. Following this incubation, cells were washed and resuspended in FACS buffer (PBS containing 5% Hyclone serum). APC-positive cells were quantified using a BD LSR II and FACSDiva.

4.7.3. Real-time PCR throughout EB differentiation

hESCs transduced with the indicated genes were gently trypsinized and placed in a low-attachment 6-well dish at a concentration of 1×10^6 cells/well in hES cell medium without bFGF. Cells were left undisturbed for the first 96 hours to allow for formation of individual EBs and media was changed every 48 hours thereafter. A fraction of EBs were collected at the indicated times and used as a source of RNA.

RNA was harvested using a Qiagen RNeasy kit. DNase I treatment was used as indicated by the manufacturer to eliminate genomic DNA. Purified RNA was used as input for the reverse transcription reaction using the Superscript III First Strand kit (Invitrogen). TaqMan assays (Applied Biosystems) were used for the following human genes and samples were prepared following manufacturer instructions: POU5F1, NANOG, VGLL4. GAPDH was used as a control. All samples were analyzed using an HT7900 machine (Applied Biosystems) using the Fast protocol. Resulting Ct values were then processed using the $\Delta\Delta C_t$ method to obtain relative changes in expression.

4.7.4. Endoderm and cardiac mesoderm differentiation

For directed differentiation into endoderm progenitors, HUES6 hES cells were treated with RPMI, 0% FBS, PenStrep, Glutamax, Activin A (100 ng/ml), and Wnt3a (20 ng/ml) for 24 hours. Cells were then switched to RPMI, 0.2% FBS, PenStrep, Glutamax, and Activin A (100 ng/ml) for 48 hours. Sox17 was stained using goat anti-Sox17 (1:500, R&D Technologies) and FoxA2 was stained using rabbit anti-FoxA2 (1:500, Upstate).

For directed differentiation into cardiac progenitors, HUES6 hES cells were treated with RPMI, 0.2% FBS, PenStrep, Glutamax and Activin A (50 ng/ml) for 48 hours. Cells were then switched to RPMI, 2% FBS, PenStrep, Glutamax, Bmp4 (20 ng/ml) and Wnt3a (20 ng/ml) for 5 days with a media change on the third day. Nkx2.5 was stained using rabbit anti-Nkx2.5 (1:100, Santa Cruz Biotechnology) and Isl1 using mAb anti-Isl1 (1:100, Developmental Studies Hybridoma Bank clone 39.3F7).

4.7.5. Teratoma formation

3×10^6 to 1×10^7 hES cells overexpressing Nanog, GFP, or Vgll4 were resuspended in hES-grade Matrigel (BD) and injected under the kidney capsule of SCID-Beige mice. 30-45 days later, mice were sacrificed and the teratoma was isolated. Tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

4.7.6. shRNA knockdown

ssDNA oligos were obtained from Invitrogen for Vgll4 (V76: Hmi423276; V77: Hmi423277; V78: Hmi423278) and Nanog (N52: Hmi475552; N53: Hmi475553; N55: Hmi475555) and processed using the instructions for the BLOCK-iT Pol II miR RNAi Expression Vector Kits. shRNAs were cloned into the EF1 α -pDEST using Gateway cloning to use the same vector used in our screening strategy. Sequences were verified using Geneious. Lentiviral particles were made as outlined in Chapter 2. Cells were transduced with the lentiviral shRNA constructs as indicated in the transduction section in previous chapters and selected with puromycin. Cells were maintained in hESC medium for 7 days after selection. After this timepoint cells were collected for RNA analysis or maintained as described above for subsequent analyses.

4.7.7. Reprogramming into iPS cells

Protocol adapted from Kit Rodolfa (Eggan Lab, Harvard University).

Human fibroblasts were seeded at 30,000 cells per well in a gelatinized 6-well dish and grown overnight in MEF medium. Cells were transduced using an MOI of 10-15 each of KLF4, SOX2, and OCT4. Retroviruses (obtained from the Harvard Gene Therapy Initiative) were generated by tripartite transfection in 293T cells and concentrated by ultracentrifuge to $\sim 10^9$ viral particles/ml. Additionally, EF1 α -pHAGE lentivirus for GFP or Vgll4 was added at an MOI of 10. An additional control with no lentivirus was also included. Cells were incubated in hES cell medium containing 8 μ g/ml polybrene (Sigma Aldrich). Cells were incubated with virus for 6-12 hours and supplemented (without removing virus-containing medium) with 1 ml of MEF medium containing 8 μ g/ml

polybrene. Cells were left in this medium for a further 48 hours and then the medium was removed carefully and replaced with hES cell medium for the duration of the experiment. Subsequently, medium was replaced daily.

4.7.8. Cell cycle dynamics by flow cytometry

hES cells were trypsinized and MEF-depleted by plating on gelatin-coated plates for 45 minutes. $1 - 4 \times 10^6$ cells were resuspended in 0.5 ml PBS followed by the addition of 0.5 ml of 100% ice-cold ethanol to the cells in a drop-wise manner while vortexing. After incubation for a minimum of 20 minutes on ice, cells were harvested by centrifugation (1000 rpm for 5-7 minutes) and the ethanol was decanted. Finally, 1 ml of Propidium Iodide-RNase solution [(final concentrations 100 μ g/ml PI (Molecular Probes) + 10 μ g/ml RNase Type I-A (CONCERT, Invitrogen) in PBS)] was added to the cells. After 30 minutes of incubation, the samples were analyzed by flow-cytometry by using BD-LSRII and FACSDiva. FlowJo analysis was used to determine the relative percentage of cells in different stages of the cell cycle using the Dean-Jett-Fox model.

4.7.9. Mitotic cell assessment by immunofluorescence

Cells were washed with PBS and fixed for 30 min with a 4% paraformaldehyde solution. Cells were then blocked using 5% donkey serum and stained with the following antibodies: anti-Oct-4 (1:200, Santa Cruz Biotechnology), anti-Human Nuclear Antigen (1:100, Millipore), anti-Phospho-histone H3 (1:100, Millipore). Appropriate secondary antibodies produced in donkey and fluorescently conjugated were acquired from Molecular Probes and used at a 1:300 dilution. Incubations with primary

antibodies were done overnight at 4°C. Secondary antibodies were incubated for 1 hr at room temperature or overnight at 4°C. Cell nuclei were stained with DAPI for 20 minutes at room temperature. Image-based quantification was done using a Cellomics system.

4.7.10. Hippo pathway activation

293T cells were transfected with the TEAD reporter plasmid (obtained from the Camargo lab, Harvard University). A day before transfection, $\sim 1 \times 10^6$ were plated per well of a 24-well plate. The next day as per manufacturer's instructions, 1.5 μ l of TRANS-IT 293 (MirusBio) and 50 μ l Optimem (GIBCO) were mixed thoroughly and incubated at room temperature for 5-10 minutes. Following this incubation, 51.5 μ l of Mirus/Optimem mix was added to 0.44 μ g of DNA with very gentle mixing and incubated for 20 minutes at room temperature. After this incubation, the mix was added dropwise onto the cells and mixed gently by moving the plate back and forth. For each reaction, 0.2 μ g of TEAD reporter plasmid and 0.04 μ g of Renilla control were mixed with 0.2 μ g of the indicated DNA. In the case of co-transfections, 0.1 μ g of each gene was used. Cells were lysed 24 hours post-transfection and analyzed with Renilla Luciferase lysis buffer (Promega). 2 μ l of lysates was then aliquoted into a 96-well. Luminometer dispensed 50 μ l of assay buffer per well and integrated luminescence over 5 seconds with a 0.1 second delay.

4.7.11. *Microarray analysis*

RNA was purified from samples in quadruplicates using a Qiagen RNeasy kit and 200 ng of starting material were used as input for the Illumina TotalPrep Amplification Kit. Samples were then hybridized to an Illumina microarray. A selection of significantly different genes was analyzed using SAM. Network and gene ontology analyses were performed using GeneGO.

4.7.12. *Assessment of TGF β signaling*

TMLC cells were maintained in MEF media (described in hES culture section above). Cells were incubated with lentiviruses for GFP or Vgll4 at an MOI of 1-2 overnight. Cells were allowed to recover for 48 hours and then selected with 2 μ g/ml of puromycin for 48 hours.

Cells were then trypsinized using 0.25% Trypsin/EDTA (Gibco) and 1×10^5 of cells were plated per well of a 96-well plate in the presence of DMSO, rhTGF β -1 at 50 ng/ml (R&D Systems), 10 μ M SB-431542 (Sigma Aldrich), or a combination of 50 ng/ml TGF β -1 and 10 μ M SB-431542. Cells were incubated in treatment conditions for 24 hours and subsequently lysed using a Luciferase Assay Kit (Promega). Luminescence was measured using a luminometer.

4.8 Note regarding author contributions

Dr. Justin Annes helped with the quantification of pH3 staining and with the design of the experiment to explore a role for Vgll4 in TGF β signaling.

Experiments to determine a role for Vgll4 in the Hippo pathway were designed, performed, and analyzed in collaboration with Dr. Morvarid Mohseni (Camargo Lab, Harvard University).

References

- [1] D. ten Berge, W. Koole, C. Fuerer, M. Fish, E. Eroglu, and R. Nusse, "Wnt Signaling Mediates Self-Organization and Axis Formation in Embryoid Bodies," *Cell Stem Cell*, vol. 3, pp. 508-518, 2008.
- [2] S. Bilodeau, M. H. Kagey, G. M. Frampton, P. B. Rahl, and R. A. Young, "SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state," *Genes & Development*, vol. 23, pp. 2484-2489, November 1, 2009 2009.
- [3] M. F. Cole, S. E. Johnstone, J. J. Newman, M. H. Kagey, and R. A. Young, "Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells," *Genes & Development*, vol. 22, pp. 746-755, March 15, 2008 2008.
- [4] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, pp. 663-76, Aug 25 2006.
- [5] N. Maherali, R. Sridharan, W. Xie, J. Utikal, S. Eminli, K. Arnold, M. Stadtfeld, R. Yachechko, J. Tchieu, R. Jaenisch, K. Plath, and K. Hochedlinger, "Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution," *Cell Stem Cell*, vol. 1, pp. 55-70, Jun 7 2007.
- [6] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells," *Nature*, vol. 448, pp. 313-7, Jul 19 2007.
- [7] M. Wernig, A. Meissner, R. Foreman, T. Brambrink, M. Ku, K. Hochedlinger, B. E. Bernstein, and R. Jaenisch, "In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state," *Nature*, vol. 448, pp. 318-24, Jul 19 2007.
- [8] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, and J. A. Thomson, "Induced

- pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, pp. 1917-20, Dec 21 2007.
- [9] R. Jaenisch and R. Young, "Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming," *Cell*, vol. 132, pp. 567-82, Feb 22 2008.
 - [10] H. Darr, Y. Mayshar, and N. Benvenisty, "Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features," *Development*, vol. 133, pp. 1193-1201, March 15, 2006 2006.
 - [11] R. H. Xu, T. L. Sampsel-Barron, F. Gu, S. Root, R. M. Peck, G. Pan, J. Yu, J. Antosiewicz-Bourget, S. Tian, R. Stewart, and J. A. Thomson, "NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs," *Cell Stem Cell*, vol. 3, pp. 196-206, Aug 7 2008.
 - [12] L. A. Boyer, T. I. Lee, M. F. Cole, S. E. Johnstone, S. S. Levine, J. P. Zucker, M. G. Guenther, R. M. Kumar, H. L. Murray, R. G. Jenner, D. K. Gifford, D. A. Melton, R. Jaenisch, and R. A. Young, "Core transcriptional regulatory circuitry in human embryonic stem cells," *Cell*, vol. 122, pp. 947-56, Sep 23 2005.
 - [13] H. Qin, K. Blaschke, G. Wei, Y. Ohi, L. Blouin, Z. Qi, J. Yu, R.-F. Yeh, M. Hebrok, and M. Ramalho-Santos, "Transcriptional analysis of pluripotency reveals the Hippo pathway as a barrier to reprogramming," *Human Molecular Genetics*, January 27, 2012 2012.
 - [14] B. Zhao, K. Tumaneng, and K. L. Guan, "The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal," *Nat Cell Biol*, vol. 13, pp. 877-83, Aug 2011.
 - [15] C. Badouel, A. Garg, and H. McNeill, "Herding Hippos: regulating growth in flies and man," *Curr Opin Cell Biol*, vol. 21, pp. 837-43, Dec 2009.
 - [16] B. Zhao, L. Li, and K. L. Guan, "Hippo signaling at a glance," *J Cell Sci*, vol. 123, pp. 4001-6, Dec 1.
 - [17] K. Schlegelmilch, M. Mohseni, O. Kirak, J. Pruszk, J. R. Rodriguez, D. Zhou, B. T. Kreger, V. Vasioukhin, J. Avruch, T. R. Brummelkamp, and F. D. Camargo, "Yap1 acts downstream of alpha-catenin to control epidermal proliferation," *Cell*, vol. 144, pp. 782-95, Mar 4 2011.
 - [18] N. Nishioka, K.-i. Inoue, K. Adachi, H. Kiyonari, M. Ota, A. Ralston, N. Yabuta, S. Hirahara, R. O. Stephenson, N. Ogonuki, R. Makita, H. Kurihara, E. M. Morin-Kensicki, H. Nojima, J. Rossant, K. Nakao, H. Niwa, and H. Sasaki, "The Hippo Signaling Pathway Components Lats and Yap Pattern Tead4 Activity to Distinguish Mouse Trophoderm from Inner Cell Mass," *Developmental Cell*, vol. 16, pp. 398-410, 2009.
 - [19] I. Lian, J. Kim, H. Okazawa, J. Zhao, B. Zhao, J. Yu, A. Chinnaiyan, M. A. Israel, L. S. Goldstein, R. Abujarour, S. Ding, and K. L. Guan, "The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation," *Genes Dev*, vol. 24, pp. 1106-18, Jun 1 2010.

- [20] H.-H. M. Chen, Steven J.; Stewart, Alexandre F.R., "Vgl-4, a Novel Member of the Vestigial-like Family of Transcription Cofactors, Regulates (alpha one)-Adrenergic Activation of Gene Expression in Cardiac Myocytes," *Journal of Biological Chemistry*, vol. 279, pp. 30800-30806, July 16, 2004 2004.
- [21] B. Zhao, X. Ye, J. Yu, L. Li, W. Li, S. Li, J. D. Lin, C. Y. Wang, A. M. Chinnaiyan, Z. C. Lai, and K. L. Guan, "TEAD mediates YAP-dependent gene induction and growth control," *Genes Dev*, vol. 22, pp. 1962-71, Jul 15 2008.
- [22] D. Lai, K. C. Ho, Y. Hao, and X. Yang, "Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF," *Cancer Res*, vol. 71, pp. 2728-38, Apr 1 2011.
- [23] G. Halder and S. B. Carroll, "Binding of the Vestigial co-factor switches the DNA-target selectivity of the Scalloped selector protein," *Development*, vol. 128, pp. 3295-305, Sep 2001.
- [24] S. Dupont, L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, and S. Piccolo, "Role of YAP/TAZ in mechanotransduction," *Nature*, vol. 474, pp. 179-83, Jun 9 2011.
- [25] G. Chen, Z. Hou, D. R. Gulbranson, and J. A. Thomson, "Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells," *Cell Stem Cell*, vol. 7, pp. 240-8, Aug 6 2010.
- [26] M. Ohgushi, M. Matsumura, M. Eiraku, K. Murakami, T. Aramaki, A. Nishiyama, K. Muguruma, T. Nakano, H. Suga, M. Ueno, T. Ishizaki, H. Suemori, S. Narumiya, H. Niwa, and Y. Sasai, "Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells," *Cell Stem Cell*, vol. 7, pp. 225-39, Aug 6 2010.
- [27] M. Ohgushi and Y. Sasai, "Lonely death dance of human pluripotent stem cells: ROCKing between metastable cell states," *Trends in Cell Biology*, vol. 21, pp. 274-282, 2011.
- [28] M. Abe, J. G. Harpel, C. N. Metz, I. Nunes, D. J. Loskutoff, and D. B. Rifkin, "An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct," *Anal Biochem*, vol. 216, pp. 276-84, Feb 1 1994.
- [29] Y. Asano, H. Ihn, K. Yamane, M. Jinnin, Y. Mimura, and K. Tamaki, "Involvement of $\alpha\text{v}\beta 5$ integrin-mediated activation of latent transforming growth factor $\beta 1$ in autocrine transforming growth factor β signaling in systemic sclerosis fibroblasts," *Arthritis & Rheumatism*, vol. 52, pp. 2897-2905, 2005.
- [30] J. S. Munger, X. Huang, H. Kawakatsu, M. J. D. Griffiths, S. L. Dalton, J. Wu, J.-F. o. Pittet, N. Kaminski, C. Garat, M. A. Matthay, D. B. Rifkin, and D. Sheppard, "A Mechanism for Regulating Pulmonary Inflammation and Fibrosis: The Integrin $\text{CE}\pm\text{vCE}\leq 6$ Binds and Activates Latent TGF $\text{CE}\leq 1$," *Cell*, vol. 96, pp. 319-328, 1999.

- [31] G. M. Beattie, A. D. Lopez, N. Bucay, A. Hinton, M. T. Firpo, C. C. King, and A. Hayek, "Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers," *Stem Cells*, vol. 23, pp. 489-495, April 1, 2005 2005.
- [32] J. R. Smith, L. Vallier, G. Lupo, M. Alexander, W. A. Harris, and R. A. Pedersen, "Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm," *Developmental Biology*, vol. 313, pp. 107-117, 2008.
- [33] L. Vallier, M. Alexander, and R. A. Pedersen, "Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells," *J Cell Sci*, vol. 118, pp. 4495-4509, October 1, 2005 2005.
- [34] L. Xiao, X. Yuan, and S. J. Sharkis, "Activin A Maintains Self-Renewal and Regulates Fibroblast Growth Factor, Wnt, and Bone Morphogenic Protein Pathways in Human Embryonic Stem Cells," *Stem Cells*, vol. 24, pp. 1476-1486, June 1, 2006 2006.
- [35] J. Massague, S. W. Blain, and R. S. Lo, "TGF[beta] Signaling in Growth Control, Cancer, and Heritable Disorders," *Cell*, vol. 103, pp. 295-309, 2000.
- [36] M. Ota and H. Sasaki, "Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling," *Development*, vol. 135, pp. 4059-4069, December 15, 2008 2008.
- [37] X. Varelas, P. Samavarchi-Tehrani, M. Narimatsu, A. Weiss, K. Cockburn, B. G. Larsen, J. Rossant, and J. L. Wrana, "The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-beta-SMAD pathway," *Dev Cell*, vol. 19, pp. 831-44, Dec 14 2010.
- [38] X. Varelas, R. Sakuma, P. Samavarchi-Tehrani, R. Peerani, B. M. Rao, J. Dembowy, M. B. Yaffe, P. W. Zandstra, and J. L. Wrana, "TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal," *Nat Cell Biol*, vol. 10, pp. 837-48, Jul 2008.

Chapter 5

Concluding Discussion and Future Directions

Embryonic stem cells represent an opportunity to realize the goals of regenerative medicine. Despite great progress in the field, the medical potential of hES cells is hindered by their propensity for spontaneous differentiation, poor survival of low-density cultures, and difficulty recovering cells after freezing. In this dissertation, we devised a genetic approach to uncover genes participating in maintaining the self-renewal of hES cells. From our screening and verification efforts, we identified Vgll4, a co-transcriptional regulator with no previously described role in ES cells. We found that Vgll4 can improve hES cell survival after dissociation, a finding with potential implications for improving the efficiency of hES cultures, especially at lower densities. We also explored other possible mechanisms for Vgll4 activity and found that it could

be participating in a variety of processes that ultimately contribute to the maintenance of the embryonic stem cell state in hES cells.

Our genetic gain-of-function screen was based on the rationale that inhibition of TGF β would allow us to find genes whose overexpression could rescue inhibition of this pathway to maintain self-renewal. Nanog was identified using a similar approach in mES cells, where cells deficient for the LIF receptor were screened for genes that could still maintain the cells in feeder-free conditions and in the absence of LIF [1]. Intriguingly, many of the genes that were hits in this screen seemed to be involved in a response to stress and modulation of cell death, indicating that these conditions were also allowing us to discover genes involved in hES cell survival. This could be due to a universal stress-response for ES cells in the presence of differentiation conditions. The latter possibility is strengthened by the observation of a large increase in cell death upon differentiation induction, as observed routinely in our laboratory. This observation also supports our view that mechanisms maintaining pluripotency, inhibiting differentiation, and promoting survival and proliferation must be closely intertwined in hES cells.

Vgll4 was independently identified as a hit several times in our screen, an event with very low probability. In addition, it consistently was able to maintain hES cell colony morphology and pluripotency marker expression after five days of treatment with inhibitors for both TGF β /Activin/Nodal and FGF signaling, suggesting a potent ability to promote pluripotency and survival. Vgll4 is a co-transcriptional regulator, which could mean a more direct role in mediating cellular decisions. However, the function of Vgll4 in hES cells represented a completely open book; in mammals, Vgll4

had only been described to have a role in heart development in the mouse. In addition to that study, data from the homologous gene in *Drosophila*, *Vg*, suggested multiple avenues for a role for *Vgll4*. In *Drosophila*, *Vg* is necessary for wing development and for the proliferation of precursor cells of the wing imaginal disc [2, 3]. Studies in *Drosophila* link *Vg* to survival and proliferation. For instance, in the absence of *Vg*, cells in the presumptive region of the larval imaginal discs undergo extensive cell death, resulting in complete absence of the wing in adults [4]. *Vg* interacts with *Scalloped* (*Sd*), the *Drosophila* homolog of TEAD. Together, *Vg* and *Sd* regulate the expression of multiple genes affecting cell proliferation and differentiation of the wing [5]. Together, these studies point to a possible role for *Vg* mammalian orthologs in the survival, proliferation, and differentiation of multipotent cell populations.

A striking and consistent observation was an increase in the population-doubling rate of cells overexpressing *Vgll4*. We reasoned that this observation could result from increased cell proliferation, a decrease in cell death, or both. We began by exploring a role in promoting survival, as cell death in response to dissociation is a unique problem to hES cells with important implications for harnessing the therapeutic potential of these cells [7]. *Vgll4* promoted survival of hES cells in self-renewal conditions as well as hES cells where E-cadherin junctions were compromised. E-cadherin junctions have been reported to control upstream signaling leading to cell death through the Rho/Rock pathway in response to dissociation in hES cells [7-9]. Our data indicates that *Vgll4* is able to promote survival of hES cells by decreasing caspase activation. It would be fascinating to understand how *Vgll4* is exerting this effect and whether it is through a direct or indirect interaction with caspases, members of the Rho/Rock pathway or

other components of the apoptosis cascade. Additionally, global transcriptional analysis shows that many apoptosis and cytoskeleton genes are downregulated in the presence of Vgll4 overexpression. Many members of the apoptosis pathway are regulated post-translationally, but it is also possible that overall protein levels are lower in Vgll4 cells, leading to a weaker activation of the cell death response. Alternatively, Vgll4 could be regulating a cytoskeleton response to promote cell survival. One possibility is that Vgll4 could prevent the myosin hyperactivation that leads to cell death or that by regulating cytoskeleton genes cell-to-cell junctions are more resistant to disruption. We observed that levels of activated caspases are higher than baseline when Vgll4 is downregulated by shRNA. This result suggests that Vgll4 is necessary for regulating caspase activation in response to dissociation. However, further decreasing the levels of Vgll4 with more effective shRNAs, TALEN nucleases [10], zinc-finger nucleases [11], or traditional knockouts would allow us to explore this phenotype further and more conclusively. Additionally, this would allow us to test for an effect in the population rate –whether it is lower in the absence of Vgll4– implying an important role in the population maintenance of hES cells.

Another question stemming from the observation that Vgll4 contributes to a higher population-doubling rate is whether Vgll4 is contributing to an increase in cell cycle or altering the dynamics of cell cycle. Our initial observations have not detected a difference in terms of population distribution across the cell cycle or number of mitotic cells in cells overexpressing Vgll4. However, we believe more that more thorough analysis is needed to make a definitive conclusion. To this end, we will examine specific markers for different phases of the cell cycle at early and late timepoints after plating,

with the rationale that the effect of Vgll4 might be easier to detect when the population is just starting to expand after passaging.

The effect of Vgll4 in affecting cytoskeleton gene expression encourages us to explore whether Vgll4 is contributing to the cell-dissociation response through the Rho/Rock pathway. It would be interesting to understand if and how Vgll4 interacts in this pathway and if it could be exploited as a way of improving cell survival further from the levels achieved with Rock inhibitor Y-27632 [12]. Importantly, Vgll4 has a significant effect in increasing the colony formation efficiency from single cells. Some of the major roadblocks in hES cell biology currently lie in the lack of genetic tools similar to those available for mouse. Generating reporter cell lines would allow the optimization of differentiation protocols by providing an efficient way of identifying cells of interest. Given the low efficiency of homologous recombination, the ability to generate reporter cell lines hinges on our ability to increase survival of hES cells from clonal densities.

In this dissertation, we have concentrated on elucidating a role for Vgll4 in hES cells. However, it is possible that Vgll4 has other effects in different cell types, especially since it is expressed in a variety of adult tissues [13]. We began to explore a role for Vgll4 in the survival of normal human dermal fibroblasts and human vascular endothelial cells. The absence of an observed effect in our assays is likely due to the fact that these cells are not sensitive to dissociation. Therefore, in the context of hES cells and other specialized cell types, it would be interesting to explore whether Vgll4 is a universal regulator of survival that can protect cells from apoptosis in the presence of other death-induction mechanisms. For instance, insights into whether Vgll4 can

overcome overexpression of TNF receptor, Fas-ligand, or other apoptotic cues would be interesting and could have implications for other diseases and therapies in humans.

As part of this dissertation, we explored several mechanisms of action for Vgll4 in hES cells. We found evidence that Vgll4 could be delaying the progression of differentiation at early stages as assessed by maintenance of colony morphology and pluripotency marker expression in the presence of differentiation cues. However, we found that this delay is temporary –cells ultimately differentiate despite Vgll4 overexpression. Nevertheless, this observation together with a possible downregulation of some of these pluripotency markers upon Vgll4 knockdown could indicate a subtle but important role for Vgll4 in stabilizing the pluripotency network. We have begun exploring the gene targets for Vgll4 regulation by ChIP-Seq as well as protein-protein interactions by Mass Spectrometry in collaboration with Dr. Laurie Boyer (MIT). These results will shed more light into the mechanism of action of Vgll4 and other key regulators of its function.

Based on evolutionarily conserved interactions of Vestigial-family genes with TEAD transcription factors, we decided to look at the recently described Hippo pathway as a possible mechanism for Vgll4 functions. Many reasons point to a possible role in this pathway: the conserved interaction with TEADs, the recent studies suggesting roles for TEADs and Hippo signaling in early embryonic development [14], stem cell self-renewal and reprogramming [15, 16], and the connection between members of the Hippo pathway and cytoskeleton regulation [17]. Vgll4 has been described as a transcriptional repressor [13], and we find that it has a powerful effect in inhibiting TEAD-mediated transcription in a reporter cell line context. An immediate next step

would involve verifying a similar role in hES cells; our microarray data already shows significant downregulation of *bona fide* Hippo/TEAD gene targets. In addition, it would be interesting to test whether these genes are upregulated in response to Vgll4 knockdown and whether reporter activity increases in response to Vgll4 downregulation. Establishing a link to the Hippo pathway could establish Vgll4 as a novel regulator of the Hippo pathway and could have implications for the variety of regulatory functions that Hippo has been linked to including organ size regulation [18-22], tissue homeostasis [23], and cancer [24].

In closing, this dissertation has explored genetic approaches to understanding molecular mechanisms involved in hES cell maintenance. We have identified Vgll4 as a novel regulator in hES cell survival. This finding represents an opportunity to improve cell survival for the application of hES cells in regenerative medicine. We also conclude that Vgll4 is a gene with a complex mode of action, and propose some of the pathways worth exploring for this gene in hES cells and other cell types.

References

- [1] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, and A. Smith, "Functional Expression Cloning of Nanog, a Pluripotency Sustaining Factor in Embryonic Stem Cells," *Cell*, vol. 113, pp. 643-655, 2003.
- [2] R. Delanoue, K. Legent, N. Godefroy, D. Flagiello, A. Dutriaux, P. Vaudin, J. L. Becker, and J. Silber, "The *Drosophila* wing differentiation factor vestigial-scalloped is required for cell proliferation and cell survival at the dorso-ventral boundary of the wing imaginal disc," *Cell Death Differ*, vol. 11, pp. 110-22, Jan 2004.
- [3] S. Bray, "Drosophila development: Scalloped and Vestigial take wing," *Curr Biol*, vol. 9, pp. R245-7, Apr 8 1999.
- [4] J. A. Williams, J. B. Bell, and S. B. Carroll, "Control of *Drosophila* wing and haltere development by the nuclear vestigial gene product," *Genes & Development*, vol. 5, pp. 2481-2495, December 1, 1991 1991.
- [5] G. Halder and S. B. Carroll, "Binding of the Vestigial co-factor switches the DNA-target selectivity of the Scalloped selector protein," *Development*, vol. 128, pp. 3295-305, Sep 2001.
- [6] F. Janody and J. E. Treisman, "Actin capping protein alpha maintains vestigial-expressing cells within the *Drosophila* wing disc epithelium," *Development*, vol. 133, pp. 3349-3357, September 1, 2006 2006.
- [7] M. Ohgushi and Y. Sasai, "Lonely death dance of human pluripotent stem cells: ROCKing between metastable cell states," *Trends in Cell Biology*, vol. 21, pp. 274-282, 2011.
- [8] G. Chen, Z. Hou, D. R. Gulbranson, and J. A. Thomson, "Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells," *Cell Stem Cell*, vol. 7, pp. 240-8, Aug 6 2010.
- [9] M. Ohgushi, M. Matsumura, M. Eiraku, K. Murakami, T. Aramaki, A. Nishiyama, K. Muguruma, T. Nakano, H. Suga, M. Ueno, T. Ishizaki, H. Suemori, S. Narumiya, H. Niwa, and Y. Sasai, "Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells," *Cell Stem Cell*, vol. 7, pp. 225-39, Aug 6 2010.
- [10] D. Hockemeyer, H. Wang, S. Kiani, C. S. Lai, Q. Gao, J. P. Cassady, G. J. Cost, L. Zhang, Y. Santiago, J. C. Miller, B. Zeitler, J. M. Cherone, X. Meng, S. J. Hinkley, E. J. Rebar, P. D. Gregory, F. D. Urnov, and R. Jaenisch, "Genetic engineering of human pluripotent cells using TALE nucleases," *Nat Biotech*, vol. 29, pp. 731-734, 2011.
- [11] M. Isalan, "Zinc-finger nucleases: how to play two good hands," *Nat Meth*, vol. 9, pp. 32-34, 2012.
- [12] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J. B. Takahashi, S. Nishikawa, K. Muguruma, and Y. Sasai, "A ROCK inhibitor permits

- survival of dissociated human embryonic stem cells," *Nat Biotechnol*, vol. 25, pp. 681-6, Jun 2007.
- [13] H.-H. M. Chen, Steven J.; Stewart, Alexandre F.R., "Vgl-4, a Novel Member of the Vestigial-like Family of Transcription Cofactors, Regulates (alpha one)-Adrenergic Activation of Gene Expression in Cardiac Myocytes," *Journal of Biological Chemistry*, vol. 279, pp. 30800-30806, July 16, 2004 2004.
 - [14] N. Nishioka, K.-i. Inoue, K. Adachi, H. Kiyonari, M. Ota, A. Ralston, N. Yabuta, S. Hirahara, R. O. Stephenson, N. Ogonuki, R. Makita, H. Kurihara, E. M. Morin-Kensicki, H. Nojima, J. Rossant, K. Nakao, H. Niwa, and H. Sasaki, "The Hippo Signaling Pathway Components Lats and Yap Pattern Tead4 Activity to Distinguish Mouse Trophectoderm from Inner Cell Mass," *Developmental Cell*, vol. 16, pp. 398-410, 2009.
 - [15] I. Lian, J. Kim, H. Okazawa, J. Zhao, B. Zhao, J. Yu, A. Chinnaiyan, M. A. Israel, L. S. Goldstein, R. Abujarour, S. Ding, and K. L. Guan, "The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation," *Genes Dev*, vol. 24, pp. 1106-18, Jun 1 2010.
 - [16] H. Qin, K. Blaschke, G. Wei, Y. Ohi, L. Blouin, Z. Qi, J. Yu, R.-F. Yeh, M. Hebrok, and M. Ramalho-Santos, "Transcriptional analysis of pluripotency reveals the Hippo pathway as a barrier to reprogramming," *Human Molecular Genetics*, January 27, 2012 2012.
 - [17] S. Dupont, L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, and S. Piccolo, "Role of YAP/TAZ in mechanotransduction," *Nature*, vol. 474, pp. 179-83, Jun 9 2011.
 - [18] F. D. Camargo, S. Gokhale, J. B. Johnnidis, D. Fu, G. W. Bell, R. Jaenisch, and T. R. Brummelkamp, "YAP1 increases organ size and expands undifferentiated progenitor cells," *Curr Biol*, vol. 17, pp. 2054-60, Dec 4 2007.
 - [19] C. Badouel, A. Garg, and H. McNeill, "Herding Hippos: regulating growth in flies and man," *Curr Opin Cell Biol*, vol. 21, pp. 837-43, Dec 2009.
 - [20] L. Zhang, F. Ren, Q. Zhang, Y. Chen, B. Wang, and J. Jiang, "The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control," *Dev Cell*, vol. 14, pp. 377-87, Mar 2008.
 - [21] B. Zhao, K. Tumaneng, and K. L. Guan, "The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal," *Nat Cell Biol*, vol. 13, pp. 877-83, Aug 2011.
 - [22] B. Zhao, X. Ye, J. Yu, L. Li, W. Li, S. Li, J. D. Lin, C. Y. Wang, A. M. Chinnaiyan, Z. C. Lai, and K. L. Guan, "TEAD mediates YAP-dependent gene induction and growth control," *Genes Dev*, vol. 22, pp. 1962-71, Jul 15 2008.
 - [23] K. Schlegelmilch, M. Mohseni, O. Kirak, J. Pruszk, J. R. Rodriguez, D. Zhou, B. T. Kreger, V. Vasioukhin, J. Avruch, T. R. Brummelkamp, and F. D. Camargo, "Yap1

- acts downstream of alpha-catenin to control epidermal proliferation," *Cell*, vol. 144, pp. 782-95, Mar 4 2011.
- [24] D. Lai, K. C. Ho, Y. Hao, and X. Yang, "Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF," *Cancer Res*, vol. 71, pp. 2728-38, Apr 1 2011.